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Approximately a quarter of breast cancer patients lack estrogen receptor (ER) expression and respond poorly to hormonal treatment. Absence of ER expression is associated with lack of transcript, methylation of the CpG island in the promoter region of this gene, and increased DNMT activity. This study addresses the hypothesis that specific inhibition of DNMT1 by antisense oligonucleotides (DNMT1 ASO) is sufficient to re-express ER in ER- human breast cancer cell lines. Significant growth reduction was observed in two ER- cell lines after 48 hr exposure to DNMT1 ASO. DMT1 expression was blocked as detected by Western whereas mutated DNMT1 ASO had no effect. Transcriptional re-expression of ER as well as other methylation-silenced genes such as PR cyclin D2 and retinoic acid receptor β was observed. ER protein expression was observed after ASO treatment. However, methylation specific PCR indicated that ER promoter CpG islands were onl6y partially demethylated. Our results suggest that specific DNMT1 inhibition alone is sufficient to re-express ER in human breast cancer cell line. This might be an alternative anti-cancer strategy for ER- human breast cancer.

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Introduction

One-quarter of all human breast cancers lack both estrogen receptorα (ERα) and progesterone receptors (PR). In general, these tumors are associated with poorer differentiation, higher growth fraction, and worse clinical outcome than ER+/PR+ breast cancer. Also, these cancers are estrogen-independent and rarely respond to hormonal therapy with agents like the antiestrogen tamoxifen. Recent findings suggest that epigenetic changes may be important for tumor initiation and progression. Abnormal methylation of CpG islands has been associated with inhibition of expression for a variety of tumor suppressor genes, including estrogen receptor α gene. A CpG island in the promoter region of the ER gene is extensively methylated in ER-negative breast cancer cells, but is unmethylated in normal breast cells. Furthermore, expression of the major enzyme which catalyzes cytosine methylation, DNA methyltransferase1 (DNMT1) is significantly elevated in ER-negative breast cancer cell lines compared to ER-positive lines. Thus, DNMT1 may play a role in blocking ER expression during progression to an aggressive, hormone insensitive phenotype.

Our study tests the hypothesis that specific inhibition of DNMT1 by antisense oligonucleotides is sufficient to re-express ERα as well as other critical breast cancer genes in ER-/PR- human breast cancer cell lines. Defining the epigenetic regulatory role of DNMT1 in breast cancer could greatly enhance our understanding of the basic changes involved in the disease as well as search for new therapeutic approaches for this the most common cancer in American women.

Body of Report

On January 1, 2000, Lan Yan, MD, Ph.D, became the principal investigator for this grant with departure of the former principal investigator, Sharyl Nass, Ph.D, to take a scientific position at the Institute of Medicine. The first part of research, expression of DNA methyltransferase and the cell cycle in human breast cancer cells, and E-cadherin 5'CpG aberrant methylation in correlation with malignant progression in human breast cancer, was done by Dr. Nass. The second part of research, specific inhibition of DNMT1 by antisense oligonucleotides induces transcriptional re-expression of estrogen receptor α in ER α-negative breast cancer cell lines, was done by Dr. Yan.

We observed that DNMT1 expression was tightly correlated with S phase fraction in ER-positive cells, while ER-negative cells expressed DNMT1 throughout the cell cycle. Thus breast cancer cells may acquire characteristics that allow them to excape normal cell cycle-dependent regulatory controls on DNMT1 expression during the process of tumor progression. Fixed nuclei were analyzed by flow cytometry following immunohistochemical staining of DNMT1 protein with a rabbit polyclonal antibody and a FITC conjugated secondary antibody, and chemical staining of the DNA with propidium iodide. We also found that the level of P21, which disrupts DNMT1 binding to PCNA, was inversely correlated with DNMT1 levels in breast cancer cells. In addition, activation of peptide growth factor signaling pathways, which is common in breast cancers, led to increased DNMT1 expression in ER-positive MCF7 cells without a concomitant change in S phase fraction. Furthermore, acquisition of an estrogen independent phenotype in MCF7 cells, even in conjunction with elevated DNMT1 expression, was not inherently accompanied by aberrant methylation of the ER gene.

These data imply that multipe steps are required for de novo methylation of the ER CpG island.

As a first step toward understanding the relationship between malignant progression and aberrant methylation of ER CpG islands, we elaluated a total of 111 ductal brast carcinomas for the incidence of ER CpG island methylation in in situ (DCIS) invasive, and metastatic lesions. We concluded that methylation of the ER CpG island can occur early during progression. About 30% of DCIS samples examined show evidence of such methylation. In addition, the incidence of aberrant ER CpG island methylation increases with progression from DCIS to invasive carcinoma to metastatic disease. Furthermore, abberrant methylation of the Er gene was a specific event – it does not necessarily take place concurrently with methylation of other CpG islands. Hwever, coincident methylation of the ER and E-cadherin genes increased with progresssion from DCIS t metastatic breast cancer.

In order to elucidate the specific role of DNMT1 in epigenetic regulation of ER gene expression, Two ER-negative human breast cancer cell lines, MDA-MB-231 and Hs578t, were selected as model systems and treated with DNMT1 antisense oligonucleotides. A DNMT1 antisense oligonucleotide (ASO98), an 18 mer targeting the 3'-untranslated region (UTR) of DNMT1 mRNA, as well as its missense control (ASO207) were synthesized by The Midland Certified Reagent Company. Cells were grown in the presence of no treatment; vehicle only (Sham); DNMT1 ASO98 (100nM x 3 days for MDA-MB-231 cells or 150 nM x 3 days for Hs578t cells) carried by lipofectin (Gibco)), or ASO207 (same treatment as ASO98). Significant growth reduction was observed for both ASO98-treated cell lines. Some growth inhibition was also observed

for both mutated ASO207-treated cell lines, possibly due to the non-specific effects of ASO. DNMT1 expression was blocked within 48 hr –96 hr of exposure to DNMT1 ASO98 as detected by RT-PCR, Western blot and DNMT enzymatic assay whereas mutated DNMT1 ASO207 had no effect. Re-expression of ERα, PR, Retinoic Acid Receptor β (RARβ), and Cyclin D2 (CD2) mRNA was observed in both cell lines as detected by RT-PCR. Re-expression of ER was also observed by Western blot analysis by use of polyclonal antibody. ERα promoter CpG methylation status was assessed by methylation specific PCR (MSP) – a technique designed to analyze methylation of CpG dinucleotides across the entire CpG island. Four sets of primers (ER1, 3,4 and 5) were used to amplify overlapping fragments of CpG island in ERα promoter region. ERα - positive MCF-7 cells were used as unmethylated control. Distilled water instead of bisulfite-treated genomic DNA was used as MSP-negative control. Cells treated with ASO98 showed the possibility of partial demethylation pattern in ER5 primer set. These results suggested that the re-expression of ERα by specific DNMT1 inhibition requiring partially CpG demethylation. Recent studies indicated that besides maintaining CpG methylation, DNMT1 can also form a repressive transcription complex at replication foci with histone deacetalases (HDACs) and a newly identified protein, DMAP1 (DNMT1 associated protein), at the non-catalytic amino terminus of DNMT1. Whether the re-expression of ERα by ASO98 is through the inhibition of DNMT1 expression with resulting disruption of the repressive transcription complex at ER promoter region needs future investigation.

1. Appendix

Reportable outcomes:

Two abstracts were presented at the AACR meeting:

1. **Nass JS**, Davidson NE. Proc. Am. Assoc. Cancer Res. 40:514-515, 1999.
2. **Yan L**, Nass JS, and Davidson NE. Effects of DNMT1 antisense oligodeoxynucleotide on steroid receptor expression in breast cancer cell lines. Proc. Am. Assoc. Cancer Res. 42:849, 2001.

Six manuscripts were published:

1. **Nass SJ**, and NE Davidson. The biology of breast cancer. Hematology/Oncology Clinics of North America, 13(2): 311-332, 1999.
2. **Nass SJ**, AT Ferguson, D El-Ashry, W Nelson, and NE Davidson. Expression of DNA methyl-transferase (DMT) and the cell cycle in human breast cancer cells. Oncogene 18: 7453-7461, 1999.
3. **Nass SJ**, JG Herman, E. Gabrielson, PW Iversen, FF Parl, SB Baylin, NE Davidson, and JR Graff. Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer. Cancer Res. 60: 4346-4348, 2000.
4. Yang X, Fergason AT, **Nass SJ**, Philips DL, Butash KA, Wang SM, Herman JG, and Davidson NE. Transcriptional activation of estrogen receptor α in human breast cancer cells by histone deacetylase inhibition. Cancer Res. 60:6890-6894, 2000.

5. **Yan L**, Yang X, and Davidson NE. Role of DNA methylation and histone acetylation in steroid receptor expression in breast cancer. J. Mammary Grand Biol. Neoplasia 6(2): 183-192, 2001.

6. Yang X, **Yan L**, and Davidson NE. DNA methylation in breast cancer. Endocrine-related Cancer 8:115-127, 2001.

One manuscript (primary data) is currently in preparation for submission in the fall of 2001.

Yan L, Philips D, Sharyl NJ, Herman JG, Nelson W, and NE Davidson. Specific inhibition of DNMT1 by antisense oligonucleotides induces transcriptional re-expression of estrogen receptor α in ERα negative breast cancer cell lines (in preparation).

List of personnel receiving pay from the research effort:

Sharyl J. Nass, Ph.D (1999);

Lan Yan MD, PhD (2000-2001).

ADVANCES IN BREAST CANCER THERAPY 0889-8588/99 $8.00 + .00

THE BIOLOGY OF BREAST CANCER

Sharyl J. Nass, PhD, and Nancy E. Davidson, MD

Breast cancer is the most frequently diagnosed cancer among women in the western world, with approximately 180,000 new cases identified annually in the United States alone. It is currently a leading cause of cancer mortality in women, second only to lung cancer. The ultimate goal in studying breast cancer biology is to reduce mortality by identifying women at risk for the disease, predicting the prognosis of existing disease, and predicting response to different therapies. This article focuses primarily on the latter two.

Many of the current therapies for breast cancer are standard cytotoxic agents which are used to treat a variety of cancer types. However, one of the most widely used and effective agents in the battle against breast cancer is the anti-estrogen, tamoxifen. The efficacy of this comparatively nontoxic hormonal therapy is based on the specific biology of breast cancer. About two thirds of breast tumors express the estrogen receptor-α (ER); many of these tumors are dependent on estrogen for

Part of this work was supported by the National Cancer Institute, the United States Army Research and Materiel Command, and the Susan G. Komen Breast Cancer Foundation.

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growth and survival and thus respond to treatment with anti-estrogens. Unfortunately, the remaining one third of breast cancers which are ER-negative at the time of diagnosis generally do not respond to endocrine therapy. Acquired resistance to tamoxifen in ER-positive tumors is also quite common. For these patients, there is clearly a need for new and better treatment options. It is hoped that improving the understanding of the basic biology of breast cancer will lead to the identification of new targets for the treatment or perhaps even to the prevention of breast cancer. Ideally, novel therapeutic or prophylactic agents would specifically target critical biological pathways in breast tumor cells.

During the process of breast tumorigenesis, mammary cells undergo numerous genotypic and phenotypic changes that allow the cells to bypass the normal controls of tissue homeostasis. Breast tumorigenesis requires a variety of genetic changes, such as activation or amplification of oncogenes or loss of tumor suppressor genes. Progression of the tumor to an aggressive, metastatic cancer depends on additional changes that permit invasion, migration, angiogenesis, and evasion of the immune system. Changes that promote genetic instability may also play a critical role in breast tumor progression, especially given the recent discovery that the protein products of the familial breast cancer genes, BRCA1 and BRCA2, associate with the DNA repair machinery of the cell. Many sporadic breast cancers also show altered expression of these genes. Although all these topics are of critical importance, they are beyond the scope of this article and are well reviewed elsewhere.[7, 9, 22, 26, 27, 34, 74]

This article focuses on the hormonal (endocrine, paracrine, and autocrine) regulation of breast development and the way that abrogations in those pathways may contribute to breast tumorigenesis by promoting inappropriate growth and survival of breast epithelial cells. Special attention is paid to how the pathways relate to or interact with estrogen signaling, because anti-estrogens have already proven themselves to be effective in the prevention and therapy of some breast cancers. Determining whether other hormonal pathways may play a role in inherent or acquired resistance to tamoxifen could lead to novel therapies that could be used either in combination with tamoxifen or after failure of tamoxifen treatment.

NORMAL MAMMARY GROWTH AND DEVELOPMENT

In order to understand how breast cancer cells differ from their normal counterparts, it is important to understand first the biology of the normal mammary gland. Unlike most tissues of the body that commonly complete growth and development during embryonic or juvenile phases

of life, mammary tissue exhibits maximum growth potential during specific reproductive-associated cycles in adult life. The mammary gland experiences multiple cycles of proliferation, differentiation, and regression resulting from repeated reproductive cycles and gestation periods.[11, 22, 23, 43]

At birth, the mammary gland consists of a primary duct and a few branching ducts within a fat pad. Between birth and puberty, mammary tissue undergoes hormone-independent isometric growth, primarily from an increase in stromal tissue. With the onset of ovarian activity, the mammary gland goes through a phase of allometric growth. Stem cells at the epithelial end bud tips rapidly divide, differentiate, and arrest in G_0 leading to elongation and branching of the ducts. Classical endocrine ablation/replacement experiments demonstrated that ductal growth requires estrogen and either prolactin or growth hormone. Mice that lack ER because of homozygous deletion (ER knockout mice) develop only vestigial ducts at the nipples, confirming the requirement for ER function in duct formation.[51] At sexual maturity, ductal development essentially stops, although the end bud epithelial tissue continues to respond to the cyclic hormonal stimuli of the menstrual cycle with alternating rounds of proliferation and apoptosis. The greatest increase in mitotic index occurs during the luteal phase of the cycle, suggesting a role for progesterone.

During pregnancy, high levels of estrogen, progesterone, and prolactin promote growth of the ducts and formation of lobuloalveolar structures. Estrogen and progesterone act synergistically to stimulate lobuloalveolar development, resulting in part from the positive regulation of progesterone receptor (PR) expression by estrogen. Experiments with mice carrying null mutations for either progesterone or prolactin receptors have confirmed that alveolar development is dependent on the function of these receptors.[12, 82] In addition to being mitogenic, progesterone inhibits initiation of lactogenesis during this stage of development. As pregnancy progresses to term, cell proliferation slows, and epithelial cells differentiate in preparation for lactation.

The decrease in hormone levels and increased intramammary pressure at the time of weaning initiates involution. During the first few days, the basement membrane of the alveoli begins to break down, and extensive cell death via apoptosis ensues. When involution is complete, only a highly branched ductal system with some alveoli remains.

In addition to classical endocrine hormones, a number of tissue growth factors are known to influence normal mammary cell growth and function (Fig. 1). For example, epidermal growth factor (EGF), transforming growth factors-β (TGF-βs), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), and related peptides have been implicated in the growth, development, and differentiation of mammary

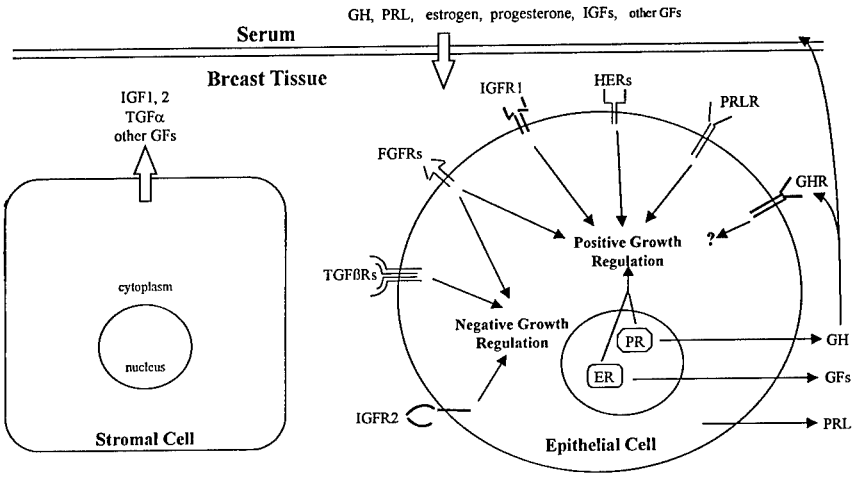

Figure 1. Some of the hormonal factors and their receptors that positively and negatively modulate normal or malignant breast epithelial cell growth and survival. Receptors for peptide hormones and growth factors are found in the plasma membrane, whereas steroid hormone receptors reside in the nucleus. There is considerable cross talk among the various pathways, and between stromal and epithelial cells. Although not shown, stromal cells also express some of the receptors found in the epithelial cells. GFs = growth factors; GH(R) = growth hormone (receptor); PRL(R) = prolactin (receptors); TGFα = transforming growth factor α; IGFR1 = insulin-like growth factor receptor-1; IGFR2 = insulin-like growth factor receptor-2; TGFβRs = transforming growth factor β receptors; FGFRs = fibroblast growth factor receptors; HERs = human EFG receptor family.

cells. In fact, it has been widely postulated that some of the effects of estrogen on breast epithelium may be indirect and dependent upon its ability to promote the paracrine or autocrine effects of growth factor pathways via up-regulation of growth factor secretion and/or receptor expression. The specific contributions of the various growth factors will be discussed in more detail later.

STEROID HORMONES IN BREAST CANCER

As described previously, the ovarian steroid hormones, estrogen and progesterone, are essential for normal mammary growth and development. Both interact with nuclear receptors that act as gene transcription factors. Two ER genes have been identified. The first, ERα, was cloned from a human breast cancer cell line and is expressed in the normal mammary gland. Recently, a highly homologous gene, ERβ, was cloned from prostate tissue.[52] Although there is preliminary evidence to suggest that ERβ may be expressed at low levels in some normal and malignant breast cells,[112] the contribution of this expression to the growth

or survival of either normal and tumorigenic breast cells is completely undefined at present. Thus, for simplicity, ERα will be referred to as ER. A single progesterone receptor (PR) gene has been identified, but at least two protein isoforms are expressed.

Estrogen

Estrogen is thought to play a significant role in the development, progression, treatment, and outcome of breast cancer.[1, 16, 33, 41, 50, 63, 86] Three consistently documented risk factors for breast cancer (age at menarche, age at menopause, and age at first pregnancy) are associated with the most dramatic physiological changes in estrogen secretion during a woman's lifetime. About two thirds of primary breast tumors are ER-positive. Tumors that express ER tend to grow more slowly, are more highly differentiated, and are associated with longer disease-free survival than tumors which lack ER. Clinical outcomes of breast cancer are related to ER expression, because ER-positive tumors are more responsive to endocrine therapy with antiestrogens such as tamoxifen.[24] Preliminary clinical results now indicate that tamoxifen and a related compound, raloxifene, may be useful for the prevention as well as for the treatment of breast cancer.[18, 46, 118]

In vitro, estrogen stimulates cell cycle progression in ER-positive breast cancer cells, but this action is temporally limited to early G_1 phase, suggesting that estrogen modulates expression or activity of G_1 regulatory proteins.[106] Similarly, anti-estrogens block proliferation during the same window of the cell cycle, with a concomitant decrease in expression of the G_1 cyclins D1 and E, and with reduced phosphorylation of Rb-1, the major target of G_1 cyclin–associated kinases.[84] Such observations suggest that overexpression of those cyclins, which is common in human breast cancer specimens,[106] could potentially interfere with the efficacy of anti-estrogen therapy. In vivo, anti-estrogens inhibit tumor growth, in conjunction with a reduced S-phase fraction.

In addition to its role as a mitogen, estrogen can also function as a survival factor for ER-positive breast tumor cells because regression of ER-positive xenograft tumors following estrogen ablation is associated with the induction of apoptosis.[55] The antiapoptotic protein, Bcl-2, is commonly expressed in human breast cancer, and that expression is associated with ER-positive tumors,[92] suggesting that estrogen may promote survival by regulating Bcl-2 expression. In ER-positive breast cancer cell lines grown in vitro, estrogen increases Bcl-2 levels without affecting expression of Bax, a proapoptotic protein.[107, 115] Furthermore, estrogen-induced increases in Bcl-2 are significantly inhibited by anti-estrogens.

Unfortunately, many ER-positive cancers eventually become resis-

tant to anti-estrogen therapy. Defining the biological mechanisms that allow breast tumor cells to survive and grow in an estrogen-independent manner could be very helpful in overcoming one of the major obstacles to effective breast cancer therapy. In most cases, the tumor cells continue to express ER but are no longer responsive to either estrogen or tamoxifen. Several possible mechanisms may contribute to this progression, including ligand-independent activation of ER, the expression of variant or mutant forms of ER, and altered expression of downstream estrogen targets.[30] However, up to one third of recurrent tumors arising from ER-positive primary tumors are ER-negative.[54]

Because estrogen promotes the growth and survival of normal breast tissue as well as ER-positive breast cancer cells, it may seem paradoxical that ER-negative tumors have a worse clinical outcome, but it has been hypothesized that ER-negative tumors are more aggressive because they have acquired the ability to bypass the ER pathways for growth and survival. In fact, re-expression of the estrogen receptor in ER-negative breast cancer cells via transfection with an ER-expression construct results in an inhibition of cell growth and tumorigenicity.[36, 45, 119] Those results suggest that loss of ER expression or function may be an important step in the progression of some breast tumors. The molecular mechanism underlying the loss of ER gene expression is not well understood, but most ER-negative cell lines and breast cancers lack ER mRNA as well as protein.[57] The absence of ER mRNA expression in those tumors does not result from detectable mutations, deletions, or other gross structural alterations in the ER gene, suggesting that inhibition of ER gene transcription is a likely mechanism. Loss of gene transcription in the absence of mutations could be explained by epigenetic modifications that do not result in a change in the primary DNA sequence. One such mechanism that may block transcription of a gene is methylation of cytosine-rich areas, termed CpG islands, in the 5′ regulatory region of the genes. CpG dinucleotides occur relatively infrequently throughout most of the mammalian genome, and most sites are methylated. In contrast, CpG islands are generally found only in gene-promoter regions and are usually unmethylated in normal adult tissues, with the exception of transcriptionally silent genes on the inactive X chromosome[10] and selected genes that are parentally imprinted to silence expression of one allele.[62] The unmethylated status of CpG islands appears to be essential for transcription, since methylation can block transcription of downstream sequences.[10, 13, 62] Anomalous patterns of DNA methylation are common in tumor cells, and many studies indicate that a variety of tumor suppressive genes are hypermethylated and transcriptionally inactive in cancer.[56]

The ER gene has a CpG island in its promoter and first exon that is extensively methylated in ER-negative breast cancer cells but remains

unmethylated in all normal tissues examined.[58, 59, 85] Using a very sensitive methylation-specific polymerase chain reaction (PCR) method, the authors have also detected ER gene methylation in a fraction of ER-positive tumors, suggesting that heterogeneity within tumor cell populations could potentially shed light on the etiology of ER-negative recurrent tumors arising from ER-positive tumors.[58] Detection of ER gene methylation in primary human breast tumors suggests that this mechanism of gene silencing could contribute to the loss of ER expression and thus to hormone resistance in breast cancer. It is not clear whether DNA methylation is the initiating or the integrative event in gene inactivation, but because the primary DNA sequence is not altered by methylation, it may be possible to reactivate gene expression by altering the methylation status. Unlike mutation, DNA methylation is reversible. Indeed, treatment of ER-negative cells with an inhibitor of DNA methylation (5-azacytidine or derivatives) resulted in demethylation within the ER CpG island and restored ER gene expression.[31] The receptor protein was functionally active, as demonstrated by its ability to activate transcription of estrogen-responsive genes.

Progesterone

The presence or absence of PR expression is an important indicator of the prospect for response to endocrine therapy. About 50% of all ER-positive breast tumors are also positive for PR. These double-positive tumors exhibit the highest response rate to endocrine therapy (about 75%), whereas less than one third of ER-positive/PR-negative tumors initially respond.[63] This finding may simply reflect the fact that ER is a key transcription factor for the expression of PR.[91] Thus, lack of PR expression in ER-positive tumors may be indicative of a nonfunctional or aberrantly functioning ER that is not likely to be affected by antiestrogens. By the same logic, it is not surprising that nearly all ER-negative tumors also lack PR and rarely respond to tamoxifen. As with the loss of ER expression, the loss of PR expression has been associated with hypermethylation in the regulatory region of the gene, but, again, it is not known whether methylation precedes or follows transcriptional inactivation.[59]

A specific role for PR in breast cancer, distinct from that of ER, has been somewhat difficult to define, but targeting the activity of this receptor can alter tumor growth.[67, 99] Curiously, both antiprogestins and supraphysiological doses of progesterone have been reported to inhibit the growth of breast cancer. In vitro, progestins produce a biphasic growth response in PR-positive breast cancer cells, with an initial increase in cell cycle progression followed by growth arrest.[106] As in the

case of estrogen, cells are sensitive to the action of progestins only in the G_1 phase of the cell cycle and respond with changes in cyclin D1 expression. The initial growth spurt is accompanied by a transient increase in cyclin D1 expression, and the long-term growth arrest is associated with decreased cyclin D1 expression, as well as with inhibition of G_1-specific cyclin-dependent kinase (cdk) activity and with a reduction in Rb-1 phosphorylation. Exposure to the antiprogestin, RU486, which can reduce tumor cell growth in vivo and in vitro, leads to Rb-1 hypophosphorylation and growth inhibition but does not target cyclin D1. Rather, cell cycle arrest by that agent is accompanied by a decrease in cyclin D3 expression. In the case of antiprogestins, it has been proposed that the G_0/G_1 cell cycle arrest is associated with a differentiation pathway, as the tumor cells form dysplastic secretory glandular structures following treatment.[68]

It is not known whether progestins can also act as survival factors for breast cancer cells, but progesterone can inhibit apoptosis in the normal, involuting mammary gland.[29] In contrast, progesterone antagonists promote apoptosis, and hence tumor regression, in xenograft breast cancer models.[69]

Recently, a new mechanism has been proposed for the mitogenic actions of progesterone on breast tissue during the luteal phase of the reproductive cycle.[72, 95] Progestins induce growth hormone (GH) production in focal areas of hyperplastic mammary epithelium, with a concomitant increase in serum GH. Furthermore, antiprogestins block the progestin-dependent expression of mammary GH. Although it remains to be proven whether this local synthesis of GH is responsible for breast cell proliferation, both the systemic and local increases in GH levels have the potential to promote mammary growth, as will be discussed later. Because breast tumors have also been shown to express GH, this mechanism could also play a role in the regulation of breast tumor growth.[71, 111]

PEPTIDE HORMONES AND GROWTH FACTORS IN BREAST CANCER

The Epidermal Growth Factor Family

The EGF family includes four transmembrane tyrosine kinase receptors, HER-1 or EGFR, HER-2, -3, and -4), and several growth factors including EGF, TGF-α, amphiregulin, and cripto-1. These paracrine factors are produced during the proliferative phases of ductal and lobuloalveolar mammary development. Many aspects of mammary biology, including survival, proliferation, and differentiation, are now thought to

be modulated by the EGF family through complex receptor heterodimerization patterns.[25]

It has been proposed that TGF-α and related growth factors may mediate the stromal-epithelial interactions involved in the indirect growth response of normal mammary epithelial cells (MECs) to estrogen. When grown in vitro, MECs proliferate in response to estrogen only when cultured in the presence of stromal cells which secrete a variety of growth factors. Transforming growth factor-α is also a known autocrine factor for breast cancer cells grown in culture, and estrogen increases expression of TGF-α and amphiregulin in ER-positive cells. Furthermore, antibodies against TGF-α or its receptor (EGFR) can block estrogen induced growth of these cells.[21, 22]

Epidermal growth factor (EGF) stimulates cell cycle progression of G_1-arrested MECs through induction of cyclin expression, cdk activity, and RB-1 phosphorylation with a temporal pattern similar to that induced by serum.[73, 101] EGF and related peptides also act as MEC survival factors as well as mitogens. Transgenic mouse models have clearly demonstrated that TGF-α can block postlactational involution.[98, 102] Experiments with normal and tranformed cell lines cultured in vitro have confirmed that TGF-α and EGF can regulate the induction of apoptosis in MECs[2, 65] by elevating Bcl-x_L levels.[75] Transfection of ER-positive breast cancer cells with the receptor HER2 resulted in increased expression of Bcl-2 and Bcl-x_L and was associated with a greater resistance to tamoxifen-induced apoptosis.[53]

A variety of mouse experiments have demonstrated the ability of EGF and TGF-α to promote mammary tumorigenesis,[21, 22] and several members of this family (both ligands and receptors) are commonly overexpressed in human breast cancer.[109] About one third of breast tumors overexpress the receptors HER2 or EGFR. Overexpression is generally associated with ER-negative tumors, high S-phase fraction, and poor prognosis.[19] In cases where the receptors are overexpressed in ER-positive tumors, several small studies suggest that such overexpression may predict poor response to anti-estrogens.[77] Taken together, the data suggest that up-regulation of these growth factor pathways may be involved in the acquisition of a hormone-independent phenotype. This hypothesis has been tested by transfecting ER-positive cell lines with HER genes under the control of constitutive promoters. Cells forced to overexpress HER2 become estrogen-independent and tamoxifen resistant.[88] A similar cell line transfected with both EGFR and TGF-α was also able to grow in estrogen-depleted media in vitro.[70] These studies suggest that targeting the pathways of this growth factor receptor family may provide new therapeutic options for some patients for whom anti-estrogen therapy is not effective. An example of such an agent is a humanized monoclonal antibody (Herceptin) that targets the extracellu-

lar portion of HER2. Preliminary reports from clinical studies with Herceptin documented its efficacy against some breast cancers that express high levels of HER2, with relatively little toxicity in nontarget tissues.[15]

The Insulin-like Growth Factor Family

The IGF family consists of two growth factor ligands (IGF1 and -2), two transmembrane tyrosine kinase receptors (IGFR1 and M6P/IGFR2) and at least seven IGF-binding proteins (IGFBP). Both IGF1 and -2 interact with IGFR1 to stimulate its tyrosine kinase activity and thereby activate multiple intracellular signaling pathways that can regulate cell growth, survival, and differentiation in many diverse tissues. In contrast, IGF2, but not IGF1, binds with M6P/IGFR2, which lacks kinase activity and has not been demonstrated to activate any intracellular pathways. Insulin-like growth factor receptor 2 may play a role in regulating IGF2 activity by internalizing the growth factor and transporting it to the lysosome for degradation.[79]

Insulin-like growth factor 1 plays a central role in normal mammary gland development. At puberty, there is an increase in serum IGF1 levels as well as in local production of IGF1 by the mammary stromal tissue. A specific role for IGF2 in mammary development has not been defined, but in humans circulating IGF2 levels are quite high compared with other classes of growth factors, and IGF2 is also produced locally by breast stromal cells. Thus, it is likely to modulate the activity of mammary cells that express the IGF1 receptor. Tissue-specific overexpression of IGF1 in the mammary glands of transgenic mice inhibits postlactational involution,[76] a result similar to that observed in TGF-α transgenics. Mice transgenic for IGF2 frequently developed mammary tumors,[6] demonstrating that this signaling pathway can promote breast tumorigenesis.

Insulin-like growth factors 1 and 2 are both potent mitogens for breast cancer cells in vitro.[105] The mitogenic action of IGF1 is synergistic with estrogen, in part because estrogen up-regulates expression of IGFR1, and IGF1 signaling leads to phosphorylation (and thus to enhanced activity) of ER. Many breast cancer cell lines express IGFR1, and interference with its expression or activity leads to inhibition of both anchorage-dependent and independent growth, as well as to reduced tumor growth in some xenograft models.

It is not known what percentage of human breast tumors are dependent on IGFR1 signaling. However, the receptor is commonly overexpressed in the epithelial cells of breast tumors, and its ligands, IGF1 and IGF2, are often highly expressed by the stromal cells surrounding the tumor.[90] Furthermore, high levels of IGFR1 expression are positively

correlated with ER expression. Breast cancer cells grown in culture respond to estrogen by increasing IGFR1 expression prior to induction of proliferation, and ER-positive breast cancer cells that overexpress IGFR1 via transfection exhibit reduced dependency on estrogen and are more sensitive to low IGF concentrations. Given these observations, it is not surprising to find that treatment with anti-estrogens such as tamoxifen leads to a reduction in IGFR1 signaling and to down-regulation of IGFR1-mediated growth. Experiments with a xenograft model have clearly demonstrated that stromal IGF2 expression can also be dramatically reduced by anti-estrogen treatment.

Despite the potent mitogenic effects of the IGF system, elevated IGFR1 expression has been correlated with good prognosis in human breast tumors. Some retrospective studies have shown that high expression levels of IGFR1 are associated with longer disease-free survival and better overall survival.[60] Again, such results may seem paradoxical, but these findings may simply reflect the correlation with hormone dependence and/or an association with an early disease stage. As the tumor progresses, other pathways may become more important, or downstream targets of the IGF pathways may be activated by other means.

In contrast with the apparent tumor-promoting effects ascribed to the IGFR1 receptor, the IGFR2 has tumor-suppressing properties, consistent with its ability to down-regulate IGF2 signaling.[79] In fact, loss of heterozygosity (LOH) of the IGFR2 gene locus has been found in approximately 30% of both invasive and in situ breast cancers. In several cases, LOH was coupled with somatic mutations in the remaining allele, a common phenomenon in tumor-suppressor inactivation. In addition to its role in IGF2 degradation, IGFR2 plays a role in the activation of a growth inhibitor, proTGFβ, pointing to an additional mechanism by which this receptor may suppress tumorigenesis.

The final members of this family, the IGFBPs, are known to transport the IGFs, prolong their half-lives, and influence interactions between the IGFs and their receptors.[80] In primary breast tumors, there is a negative correlation between ER status and IGFBP3 expression. In vitro, estrogen inhibits the production of IGFBP3 by breast cancer cells, and the addition of exogenous IGFBP3 can block estrogen-stimulated proliferation. Anti-estrogens, on the other hand, have been reported to up-regulate the expression of several IGFBPs. Taken together, these observation could again suggest that down-regulation of the IGF system might potentially play a role in the efficacy of tamoxifen. However, recent findings also point to an IGF-independent mechanism by which IGFBPs, especially IGFBP3, can potently inhibit growth.[80] This effect appears to be mediated by interaction with a poorly characterized cell-surface–associated protein

and appears to play a direct role in the growth suppressing effects of such diverse agents as TGF-β and retinoic acid in breast cancer cells.

The Fibroblast Growth Factor Family

The FGF family consists of four known transmembrane tyrosine kinase receptors and at least nine ligands that interact with the receptors with varying affinities. It has been hypothesized that receptor heterodimerization may contribute to the complexity of responses regulated by this family, analogous to the EGF family. Several FGFs have been localized in the developing murine mammary gland, and normal mouse MECs require FGF for growth in culture.[22]

That several FGFs were originally identified by transformation assays suggests that they could potentially play an important role in tumorigenesis. One of the first indications that FGFs could promote mammary neoplasia came from studies with the mouse mammary tumor virus (MMTV). The genes for FGF3 and -4 are both frequent targets of proviral insertion and activation in viral-induced tumors, and overexpression of FGF3 alone in transgenic mice can induce tumor formation.[47] In vitro, many human breast cancer cell lines proliferate in response to FGFs.

Expression of several FGF ligands and receptors has been observed in normal and malignant breast tissue, but expression levels vary greatly in both, so it is difficult to assess the role of these factors in breast cancer growth or to determine whether changes in expression are contributing to tumorigenesis.[47] However, transfection of ER-positive breast cancer cells with either FGF1 or -4 results in estrogen-independent growth and resistance to anti-estrogens, both in vitro and in vivo.[47, 64, 120] Increased metastatic ability and angiogenesis in vivo were reported as well, indicating that FGFs can have complex, pleiotrophic effects on breast tumor cells. A subsequent study found that paracrine effects of FGF1 on nontumor cells could act in synergy with the mitogenic effects of estrogen, whereas autocrine FGF1 stimulation of the epithelial cancer cells was required for estrogen-independent tumor growth.[121]

In contrast to the apparent growth-promoting effects of FGF1, -3, and -4 on breast cancer cells, FGF2 has been reported to inhibit the growth of several human breast cancer cell lines. This inhibition appears to be result from a block in cell cycle progression via increased expression of the G_1 cdk inhibitor p21,[113] as well as from an induction of apoptosis following an increase in bax expression coupled with a decrease in bcl-2.[114] It was also reported that FGF2 treatment increased the sensitivity of the cancer cells to traditional chemotherapeutic agents.[113] Interestingly, two recent studies found that higher levels of FGF2 protein

in primary breast tumors were associated with longer disease-free and overall survival, adding credence to the in vitro observations that FGF2 can suppress breast tumor growth.[17, 118]

The Transforming Growth Factor-β Family

Transforming growth factor-βs (1–3) are multifunctional growth factors that interact with two interdependent serine-threonine kinase receptor subtypes. This class of peptides inhibits the growth of most epithelial cells and promotes the growth of stromal cells. In the normal mammary gland, TGF-β is a critical paracrine regulator of epithelial cell growth and regression. It potently inhibits ductal elongation during gland development[96] and promotes apoptosis during postlactational involution.[104]

In nontransformed mammary epithelial cells, TGF-β has been reported to block expression of the S-phase–promoting cyclin A protein, but to inhibit only moderately the expression of the G_1 cyclins D1, D2, and E.[101, 103] Activity of existing G_1 cyclin-cdk complexes was repressed by TGF-β–induced changes in the cdk inhibitor p27, thereby blocking Rb-1 phosphorylation and thus the S-phase transition. However, it should be noted that Rb-1 function does not appear to be an absolute requirement for growth arrest by TGF-β in breast cancer cell lines.[81]

Transforming growth factor-β can induce apoptosis as well as cell cycle arrest in normal and transformed MECs. Transgenic mice that overexpress TGF-β show increased occurrence of apoptosis in the mammary epithelium, with a subsequent lack of secretory lobule development.[44] Several studies have reported increased TGF-β expression in human breast cancer cells which have been stimulated to undergo apoptosis by a variety of factors, including cytotoxic drugs, anti-estrogens, or hormone ablation.[3, 4, 55, 67] However, it was not determined whether TGF-β secretion was required for apoptosis induction in those systems. In mammary tumor cells which overexpress the c-myc oncogene, TGF-β can promote apoptosis by blocking survival factor-dependent up-regulation of the antiapoptotic Bcl-x_L protein.[75]

Because TGF-β inhibits the growth of normal mammary epithelial cells in vivo[20, 100] and breast cancer cells in vitro,[110, 122] it was originally hypothesized that TGF-β treatment could be used to inhibit mammary tumor growth. Mice transgenic for TGF-β have demonstrated that elevated TGF-β expression can suppress mammary tumor incidence.[87] However, no antitumor effect was observed in xenograft tumor-bearing mice treated with exogenous TGF-β.[123] Furthermore, it has been demonstrated that TGF-β expression is actually higher in human breast tumors than in normal mammary tissue, and protein levels are positively correlated with disease progression.[39]

It may be advantageous for the tumor cells to produce large quantities of TGF-β because of its potential role in promoting angiogenesis[28] and invasion [97] or in suppressing the immune system.[5] The breast tumor cells must therefore develop the ability to grow in the presence of relatively high concentrations of TGF-β. A potential mechanism for resistance is mutation of the receptor or a change in receptor subtype.[93] However, a change in activity or expression of one of the downstream targets of the TGF-β intracellular signal pathway might also be effective in blocking growth inhibition.

Determining the exact mechanism by which cancer cells become resistant to TGF-β could identify novel therapeutic targets. This possibility is particularly interesting in light of the hypothesis that the therapeutic and preventative effects of anti-estrogens may be mediated at least in part by up-regulation of TGF-β.[93] One study has shown that acquisition of estrogen independence in vitro is accompanied by resistance to TGF-β.[42] If that is in fact true, then targeting TGF-β resistance could potentially restore sensitivity to anti-estrogen therapy in some patients who have become resistant to tamoxifen.

Growth Hormone and Prolactin

Growth hormone (GH) and prolactin (PRL) are closely related neuroendocrine hormones. Their transmembrane receptors (GHR and PRLR) also exhibit extensive homology and belong to a class of the cytokine receptor superfamily.[38] In fact, human GH can bind to and activate both GHR and PRLR. Several different forms of the receptors have been reported, including soluble forms consisting of only the extracellular portion which act as binding proteins. The functional significance of the various isoforms remains obscure, but a role in tissue-specific regulation of PRL function has been suggested.[8]

Pituitary-derived GH plays an important role in regulating the serum levels of IGF1 by inducing hepatic IGF1 production, and the surge of circulating IGF1 at puberty contributes to the initiation of ductal formation in the mammary gland.[48] Animal models suggest that this GH/IGF1 axis may play a role in breast tumorigenesis as well as in normal breast development. Aging primates treated with exogenous GH exhibit a dramatic increase in mammary epithelial proliferation index and gland size that is correlated with increased serum IGF1 levels.[78] Growth hormone–transgenic mice have elevated serum IGF1 levels and exhibit hyperplasia of the mammary gland, with a high frequency of breast tumor incidence.[108] In contrast, mice transgenic for a GH antagonist show ductal hyperplasia. Ablation of the GH/IGF1 axis in mice

with human breast cancer xenografts, or transplant of xenografts into GH-deficient mice, results in reduced tumor growth.

There is also considerable circumstantial evidence to suggest a role for the GH/IGF1 axis in human breast tumorigenesis.[89] For example, height is positively correlated with serum IGF1 levels and is also associated with increased breast cancer risk. Two retrospective studies have found significantly higher serum IGF1 levels in women with breast cancer than in controls, especially in premenopausal women. More recently, a study using prospectively acquired blood samples has provided more direct evidence that activity of the GH/IGF1 axis is related to risk of premenopausal breast cancer.[40] However, since GH production naturally decreases with age, it remains to be determined whether higher IGF1 levels during the premenopausal years may also influence the risk of breast cancer after menopause. In any case, this study suggests that drugs targeting the GH/IGF1 axis may have potential use for both prevention and therapy of breast cancer.

Although the mechanism is not known, adjuvant tamoxifen therapy has been reported to suppress the pulsatile secretion of pituitary GH and to reduce serum IGF levels. Whether these changes are necessary for tamoxifen's efficacy is not known, but such observations raise the question whether prevention or therapy with tamoxifen will be more effective in women with higher pretreatment IGF levels or in those who show the greatest decrease in serum IGF following tamoxifen administration.[89]

It is also interesting to note that, although GH is best known for its role in the systemic GH/IGF1 axis, it also has the potential to act directly on breast tissues because local expression of both GH and GHR has been demonstrated in the majority of normal and malignant breast tissue, as well as in a variety of benign breast lesions.[66, 71, 72, 111] Although GHR did not appear to be grossly overexpressed in malignant tissues, this potential autocrine/paracrine loop could be important for the growth or survival of breast tumor cells, perhaps by up-regulating local IGF1 production,[49] or by interaction of locally produced GH with the PRLR in the breast.

The endocrine effects of PRL on human breast tissue include the regulation of growth and differentiation of ducts and lobules, as well as the initiation and maintenance of lactation. Prolactin is also a mitogen for human breast cancer cells in culture, and anti-PRL reagents can inhibit the growth of those cells.[35, 37] Prolactin also acts synergistically with ovarian steroids to promote the growth of human breast cancer xenografts in mice.[61]

A role for PRL in rodent mammary tumorigenesis has been clearly demonstrated. Prolactin contributes directly to the etiology of both spontaneous and carcinogen-induced murine mammary carcinoma, and treat-

ment with anti–PRL agents induces a significant therapeutic response.[116] However, its role in human breast tumorigenesis is poorly defined. Prolactin has been largely discounted as a determinant in breast cancer because there is a lack of correlation between circulating PRL levels and the incidence or clinical outcome of the disease, and treatments that suppress pituitary PRL release have not been shown to improve outcome.[32] However, it is now known that PRL is produced in many extrapituitary locations, including the breast.[8] If locally produced PRL acts as an autocrine/paracrine factor, then its role in tumorigenesis may be independent of circulating PRL levels.

Both PRL and its receptor are both widely expressed in normal breast tissues and in the epithelial cells in breast tumors, confirming the potential for an autocrine/paracrine loop in these tissues.[14, 66, 83, 94] Three studies using in situ hybridization and immunohistochemistry, or reverse transcription polymerase chain reaction (RT-PCR) detected expression in nearly all samples analyzed. The fourth study using quantitative Northern analysis found a positive correlation between expression levels of PRLR and that of ER and PR in a large panel of both human breast cancer cell lines and primary tumors.[83] In the same study, the authors reported that in human breast cancer cell lines, acute treatment with progestins or long-term treatment with estrogen increased PRL receptors; addition of exogenous PRL resulted in elevated PR expression. This receptor cross-regulation may provide one explanation for the observed synergy among estrogen, progesterone, and PRL in the control of normal and malignant breast tissue growth.

SUMMARY

This article focuses on the major hormones and growth factors for which a critical role in normal mammary growth has been clearly defined. Certainly other hormonal systems and growth factors could also affect breast cancer initiation and progression, but their exact contribution to normal and/or malignant breast cell growth is poorly delineated. Examples of such factors include somatostatin, mammostatin, mammary-derived growth inhibitor (MDGI), mammary-derived growth factor-1 (MDGF-1), inhibins, activins, androgens, glucocorticoids, vitamin D, thyroid hormones, ecosinoids, and oxytocin.

Clearly, the hormonal regulation of breast cancer cell growth and survival is multifaceted and very complex. In particular, the effects of estrogens and anti-estrogens on breast cells may depend on their interaction with a wide variety of other pathways. In addition, these interactions may vary among individual breast tumors depending on other genetic changes in the tumor cells that have not been discussed here, such as oncogene activation and loss of tumor suppressors. A more

detailed understanding of how cells circumvent a dependency on these pathways is greatly needed in order to identify new biological targets and to design novel therapies for breast cancers that are resistant to anti-estrogen therapy. Such agents could be used alone or in combination with anti-estrogens to improve response to a second course of hormonal therapy.

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N-terminal region from amino acids 1 to 363. A C-terminal region of HDAC1 (aa 363–482) does not interact with hHUS1. These data suggest that hHUS1 can interact with HDAC1. Interestingly, in *S. pombe*, HUS1+p acts as a G2/M and DNA damage checkpoint factor (Kostrub *et al. Mol. Gen. Genet.* 1997, 254: 389), which suggests an additional potential involvement of HDAC1 in cell cycle regulation.

#3390 The AML/ETO fusion protein modulates the transcriptional effects of histone deacetylases, PLZF and other corepressor proteins. A. Melnick, J.J. Westendorf, S. Arai, B. Lutterbach, H.J. Ball, A. Polinger,* S.W. Hiebert and J.D. Licht. *Mount Sinai School of Medicine, New York, NY 10029 and Vanderbilt University School of Medicine, Nashville, TN 37232.*

The AML/ETO fusion product is expressed in acute myeloid leukemias which harbor the t(8;21) translocation. This chimeric protein can disrupt —possibly in a dominant negative manner - the normal function of the AML1 gene product. The ETO protein is also known to associate with a transcriptional repression complex which includes histone deacetylases (HDACs), co-repressor proteins as well as the PLZF protein — fused to the retinoic acid receptor α (RAR α) in t(11;17) promyelocytic leukemia. PLZF is a growth suppressor expressed in early myeloid cells. We have previously shown that PLZF and ETO interact physically and functionally to enhance transcriptional repression. We wished to know if the AML/ETO fusion product could (in addition to AML1) disrupt ETO and PLZF functions. We used a variety of transcriptional assays with reporters driven by GAL, PLZF and retinoic acid receptor binding sites to determine these effects. We found that i) AML/ETO is able to partially antagonize transcriptional repression mediated by PLZF. ii) AML/ETO completely abrogates the cooperative effect of PLZF and ETO. iii) AML/ETO is able to block the transcriptional cooperation between PLZF and other co-repressors such as N-CoR and SMRT. iv) AML/ETO is able to antagonize transcriptional repression mediated by HDACs 1, 2 and 3 and, v) AML/ETO can antagonize the transcriptional repression mediated by the aberrant PLZF/RAR fusion partner on its binding sites. Thus, we conclude that AML/ETO behaves as a dominant negative factor, not only for ETO but for other myeloid specific transcriptional regulators, and that this dysregulation might suggest a common pathway of leukemogenesis in several myeloid leukemias.

#3391 Cell-cycle dependent transcription of cyclin B1: role of histone acetyl transferase. Nair, A.R., Hwang A., Cheng Y., Muschel, R.J. *Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104.*

The triggering of the transition from G2 to M requires a dramatic increase in cyclin B1 protein that is brought about by increased cyclin B1 mRNA levels at G2. In order to understand the elements controlling the cell cycle regulation of transcription of the cyclin B1 gene, its promoter was cloned. The cell cycle regulatory elements were shown to reside in the 90 bases upstream from the transcription start site. Using substitution mutations we have identified two CAAT sequences within the 90 base pair region that are required for cell cycle regulation. NF-Y, a CAAT binding factor binds to these sites. NF-Y has been shown to associate with hGCN5 and P/CAF, two histone acetyl transferases. In this system also, we show that NF-Y associates with hGCN5. These observations led to the hypothesis that cell cycle regulated transcription of cyclin B 1 might be controlled by histone acetylation. The histone acetylase activity that is immunoprecipitated by anti NF-Y antibodies varies through the cell cycle, with levels 5 fold lower at the G1/S boundary than in M. Trichostatin A, a specific inhibitor of histone deacetylase, inhibits the increase in mRNA levels of cyclin B1 during cell cycle progression. This inhibition is not due to perturbation of the cell cycle by Trichostatin A. These results indicate that NF-Y associated histone deacetylase activity plays a role in the cell cycle dependent transcription of cyclin B1.

#3392 Structure-function analysis of the KRAB/KAP-1 repressor/corepressor system suggests a histone deacetylase independent mechanism of transcriptional repression. Schultz, D.C., Ryan, R.F., Friedman, J.R., Rauscher, III, F.J. *The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104.*

The KRAB domain, a 75 amino acid module encoded by approximately one-third of all Kruppel-type zinc finger proteins, is a DNA binding-dependent repression domain. Transcriptional repression directed by a KRAB domain is mediated through a novel corepressor molecule, KAP-1. The c-terminus of KAP-1 contains a novel region of amino acids, a PHD finger, and a Bromo-like domain which together are required for optimal KAP-1 mediated repression. To identify components of the KAP-1 repression complex and/or downstream effectors we have performed extensive yeast two-hybrid and biochemical analyses. The PHD finger and the Bromo-like domain were found to interact with the carboxy terminus of CHD3/Mi-2α*in vivo* and *in vitro*. CHD3 encodes a nuclear protein containing signatures for ATPase/helicase, chromodomains, and PHD fingers. Overexpression of a dominant negative mutant containing the carboxy terminus of CHD3 was observed to strongly inhibit KAP-1 mediated transcriptional repression. Biochemical analysis revealed that KAP-1 is present in potentially two different complexes of 1–2 MDa and 670 kDa apparent molecular mass, respectively. Immunoprecipitation of KAP-1 from Hela nuclear extract with two independent affinity purified polyclonal antibodies identified 8 to 9 potential associated polypeptides, none of which immuno-react with antibodies against histone deacetylase or previously described components of repression complexes. Overall these data indicate that the KRAB/KAP-1 repressor-corepressor system may participate in higher-order chromatin assembly/remodeling which appears to be independent of histone deacetylase.

#3393 Mutations in the conserved domains of histone deacetylase confer loss of enzymatic function while retaining binding to associated proteins. Kwon, P., Xu, H., Trogani, N., Cai, R., Fischer, D., Skelton, J., Cohen, D., and Sambucetti, L. *Novartis Institute for Biomedical Research, Novartis Pharmaceuticals Corporation, Department of Oncology and Department of Functional Genomics, Summit, NJ 07901.*

Chromatin modification has emerged as a major regulatory mechanism in gene activation and silencing leading to effects on cell proliferation. To elucidate the relationship between histone deacetylase (HDAC1) structure and its function, site-directed mutants were constructed within conserved as well as nonconserved domains and assayed for enzyme activity and interaction with putative associated proteins. The mutational analysis of HDAC1 revealed that changes in the conserved regions confer loss of activity while mutations in the nonconserved regions retain enzymatic function. This result suggests the involvement of the conserved residues in the active site. In addition, all mutants including the inactive ones interacted with several putative associated proteins including Retinoblastoma (Rb) protein, p107, and mSin3 implying conformational integrity of the enzyme. One inactive mutant with modifications at the putative Rb binding domain provided additional insight. Although this mutation lies in the less conserved C-terminal region, interaction with Rb and enzyme activity were abrogated. Furthermore, preliminary results indicate association of HDAC1 to itself but the functional significance of such multimerization is still under investigation. The above results demonstrate that structural integrity of the conserved domain is required for enzymatic activity although not required for interaction with associated proteins.

#3394 Evidence for a trans-acting factor in embryonic carcinoma cells that can induce epigenetic inactivation. Mitchell Turker and Phil Yates. *Center for Research on Occupational and Environmental Toxicology, L606, Oregon Health Sciences University, Portland, OR USA.*

A cis-acting methylation center (MC) upstream of the mouse *aprt* gene has been shown to direct epigenetic inactivation when placed proximal to a truncated *aprt* promoter and stably introduced into the P19 embryonic carcinoma cell line (*J Biol Chem* **270**:788–792, 1995). The embryonic cell line used for these experiments lacked both endogenous *aprt* alleles. We show here that epigenetic inactivation rarely occurred when the MC-promoter construct was stably introduced into an *aprt* deficient, differentiated version of the embryonic cell line. However, high frequency epigenetic inactivation was observed when differentiated cell lines expressing the MC-promoter construct at high levels were fused to the embryonic cells. Significantly, inactivation was not observed when the differentiated cells contained *aprt* promoter constructs lacking the MC fragment. These results suggest the presence of an embryonic factor that can interact with the MC fragment to induce epigenetic inactivation.

#3395 Growth constraint induces methylation of CpG islands in cultured normal human fibroblasts (NHF). Fanton, Christie P., Lester, Kathryn A., and Pieper, Russell O. *Brain Tumor Research Center and the Dept. of Neurosurgery, UCSF Cancer Center, San Francisco, CA 94115.*

Methylation-associated gene silencing has been noted in transformed cells and in normal cells nearing the end of their lifespan. Data presented in this study suggest that CpG island methylation can also occur in normal, non-senescent cells in response to factors that restrain growth. NHF (ATCC WI38, population doubling 22) were either serially passaged at low density or were left in high density conditions for 21 days. Following DNA isolation, bisulfite sequencing was used to analyze methylation in select regions of the CpG islands of the estrogen receptor (ER), E-cadherin (E-CAD), p16, and MGMT genes in both groups. Logarithmically growing NHF displayed little cytosine methylation (< 10% of CpGs analyzed) in the four CpG islands examined. Growth-constrained cells also showed little cytosine methylation (<10% of CpGs analyzed) in the ER, E-CAD, and p16 CpG islands. The MGMT CpG island in growth-constrained cells was, however, extensively methylated (>60% of CpGs analyzed). Cells displaying MGMT CpG island methylation were not senescent (by beta-galactosidase staining) and resumed growth following replating. Methylation in the MGMT CpG island was retained through an additional four population doublings but was lost within ten population doublings. These results suggest that conditions that restrain the growth of normal, proliferative cells may trigger genomic instability in the form of CpG island methylation. The factors that restrain growth and trigger genomic instability in vitro may resemble those found in and near solid tumors in vivo.

#3396 DNA methyl-transferase (DMT) expression and the cell cycle in breast cancer. Nass SJ, Ferguson AT, El-Ashry D, Nelson W, Davidson NE. *Johns Hopkins University, Baltimore, MD 21231, Georgetown University, Washington DC 20007.*

ER– breast cancer cells display extensive methylation of the ER gene CpG island and have elevated DMT expression compared to ER+ cells. ER+ cells expressed DMT primarily in the S phase, whereas ER– cells expressed DMT

...out the cell cycle. In addition, levels of p21(CIP1), which disrupts DMT ...to PCNA, were inversely correlated with DMT levels. The results suggest ...creased DMT expression in ER− cells is due to more complex changes ...a simple elevated S-phase fraction. To determine whether growth factor ...ays play a role in the regulation of DMT expression in breast cancer, cell ...erived from ER+ MCF-7 cells were examined. These lines all grow in ...en-free conditions as a result of selection or transfection. Among untrans... or control vector-transfected lines, DMT level was correlated with S-phase ...n similar to the original panel of ER+ cell lines. Overexpression of a ...utively active Raf kinase also led to increased DMT expression, but the ...e in expression could be fully explained by a corresponding increase in S ...fraction. Transfection with FGF1 or 4 led to increased DMT expression that ...not be accounted for by a shift in S phase fraction. The elevated DMT ...n expression in FGF transfectants was accompanied by a dramatic de... in p21, again suggesting a reciprocal relationship between these two ...ts. Although all of the MCF-7-derived cell lines examined grow independent ...rogen, all but one (Raf14c) express ER protein. Furthermore, the ER CpG ...remained unmethylated all of these cell lines, including Raf14c. Thus, ...sition of an estrogen-independent phenotype, even in conjunction with ...ed DMT levels, was not sufficient to promote ER gene silencing via CpG ...methylation.

...LL AND TUMOR BIOLOGY 19: Protein Kinases in ...mor Progression/Regression

...397 The effects of PKCα on integrin life cycle and tumor cell hapto-... Ng, T.T.C., Bastiaens, P., Humphries, M.J. and Parker, P.J. *Imperial Cancer ...earch Fund Laboratories, London and Wellcome Trust Centre for Cell-Matrix ...search, University of Manchester, United Kingdom.*

...he extracellular matrix provides both survival/proliferative and migratory sig... to cancer cells through various integrin receptors. The surface expression ...f conformation (hence ligand binding avidity) of these receptors are regulated ...intracellular events which are poorly characterized. Using fluorescence resc... ...ce transfer (FRET) and fluorescence lifetime measurements, we demonstrate ...MCF-7 cells a stable *in vivo* association between protein kinase C (PKC)α (full ...th or regulatory domain alone) and the activated form of integrin β1 subunit ...the plasma membrane as well as in intracellular vesicular structures. The active ...formation of β1 integrins is stabilized by an overexpression of PKCα. The ...ribution of activated integrins between the plasma membrane and the intra... ...ular pool is influenced by signals that activate PKCα which catalyzes the ...ternalization of integrins through recycling endocytic pathways, identical to ...ose used by the human transferrin receptor. PKCα-induced endocytosis of ...and-bound integrin receptors is sensitive to both changes in Ca^{2+} levels and ...phoinositide-3-kinase (PI3k) activity. These intracellular molecular events are ...icately linked to and possibly responsible for the cell shape changes and ...egrin-mediated haptotaxis in response to PKCα activation. The implications of ...ese findings for understanding the directional cell motility and hence metastatic ...tential of tumor cells will be discussed in relation to our recent work on the ...orescence lifetime imaging of PKC activation in human breast carcinomas.

...3398 MAP kinase stimulation by G-protein-coupled receptors requires ...ab1 mediated PI 3-kinase activation. Holgado-Madruga, M., Dieterich, R., ...oscatello, D.K., Baudry, K., and Wong, A.J. *Kimmel Cancer Institute, Thomas ...efferson University, Philadelphia, PA 19107.*

...G-protein-coupled receptors (GPCRs) have been implicated in the pathogen... ...esis of several human tumors. Stimulation of GPCRs results in a diverse number ...of physiologic processes including mitogenesis. Lysophosphatidic acid (LPA), a ...GPCR ligand, is perhaps the major mitogenic component of serum. The mitogen ...activated protein (MAP) kinase pathway is essential for GPCR-induced mitogen... ...esis. MAP kinase activation requires tyrosine kinase activity and subsequent PI ...3-kinase activity, but the molecules linking these events are still being elucidated. ...Here, we demonstrate a role for the Gab1 docking protein in GPCR activation of ...MAP kinase. Gab1 is tyrosine phosphorylated and associates with Grb2 after ...LPA, endothelin-1 and thrombin stimulation. Gab1 phosphorylation is sensitive to ...AG1478, suggesting that the EGF receptor is activated in this pathway. LPA ...stimulation results in association of phosphatidylinositol (PI) 3-kinase activity with ...Gab1. The overexpression of Gab1 augments both MAP kinase activation and ...mitogenesis, but mutation of the PI 3-kinase binding sites or deletion of the Gab1 ...pleckstrin homology (PH) domain abolishes these effects. Gab1-Grb2 association ...also depends upon the PI 3-kinase binding sites or the PH domain of Gab1. ...Furthermore, we demonstrate that the PH domain of Gab1 interacts with a ...product of PI 3-kinase, PI $(3,4,5)P_3$. These results indicate that Gab1 is an ...essential component in the GPCR-mediated activation of MAP kinase.

#3399 Differential effects of EGF and Heregulin on reversing growth inhibition mediated by anti-EGF receptor mAb C225 and by anti-HER2 mAb 4D5 (Herceptin™) in OVCA420 human ovarian cancer cells. Ye, D., Mills, G., Mendelsohn, J., and Fan, Z. *The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030.*

We previously have shown that combination treatment of OVCA420 human ovarian cancer cells with anti-EGF receptor mAb C225 and anti-HER2 mAb 4D5 (Herceptin™) resulted in additive inhibitory effects on the proliferation of these cells. In the present study, we further delineated the mechanism by which the mAbs inhibit the proliferation of these cells. Exposure of the cells to C225 and/or Herceptin™ reduced the basal levels of both MAP kinase and PI3 kinase activities. Growth inhibition mediated by C225 and/or Herceptin™ can be prevented by concurrent exposure of the cells to EGF or, to a lesser extent, Heregulin (HRG). Addition of a MAP kinase specific inhibitor PD98059 or a PI3 kinase specific inhibitor LY294002 only partially reversed the EGF-mediated protection of the cells from the antibody-induced growth inhibition; whereas the combination of PD98059 and LY294002 completely abolished the capacity of EGF to reverse antibody-induced growth inhibition. In contrast, addition of either PD98059 or LY294002 nearly completely reversed HRG-mediated protection of the cells from the antibody-induced growth inhibition. Our data suggest that C225 and Herceptin™ decreased growth of these ovarian cancer cells through inhibition of both MAP and PI3 kinase pathways. EGF is a broadly-acting growth factor and inhibition of both MAP kinase and PI3 kinase pathways is required to prevent EGF-mediated protection from the antibody-induced growth inhibition; whereas HRG is a narrowly-acting growth factor and inhibition of either MAP kinase or PI3 kinase is sufficient to abolish the capacity of HRG to protect cells from the antibody-induced growth inhibition.

#3400 Regulation of PLC-γ by a novel cytosolic docking protein, CAIR-1, in A2058 human melanoma and MDA-435 breast cancer cells. Doong, H., Blanchette, J., and Kohn, E.C. *Lab. of Path., National Cancer Institute, Bethesda, MD 20892.*

We previously reported a new PLC-γ partner protein, CAIR-1. The hypothesis that blocking nonvoltage-operated calcium entry would alter expression of proteins involved in its downstream pathways yielded discovery of CAIR-1. The current study addresses the issues of CAIR-1 expression in different cell types and how the CAIR-1/PLC-γ complex is regulated. CAIR-1 was detected in A2058 melanoma, MDA-435 breast cancer, CaOV3 and OVCAR-3 ovarian cancer cells, and human umbilical vein endothelial cells. CAIR-1 expression is demonstrated by immunohistochemical staining of colon, prostate, breast, and ovary epithelium and tumor cells, but not in normal stromal cells. CAIR-1 is tyrosine phosphorylated upon EGF stimulation. A cytoplasmic protein, CAIR-1 does not translocate to the plasma membrane after EGF stimulation. CAIR-1 co-immunoprecipitates with PLC-γ in unstimulated cells. Upon EGF stimulation, there is a log linear dose dependent reduction in PLC-γ binding to CAIR-1 (r^2=0.99, EC_{50}=55ng/ml). D-erythrosphingosine, a protein kinase C (PKC) inhibitor, was found to inhibit both basal phosphorylation of CAIR-1 and its dissociation from PLC-γ. These results indicate that the CAIR-1/PLC-γ complex is regulated by Ser/Thr phosphorylation of CAIR-1 and by the EGF pathway. Domain analysis of CAIR-1 reveals 4 PXXP putative SH3 binding domains in CAIR-1. Studies to determine which domain(s) are specific for PLC-γ binding and phosphorylation are ongoing.

#3401 Compensatory stabilization of RIIβ protein in protein kinase A-RIα antisense treated cancer cells: A novel antitumor-antisense mechanism. Cho-Chung, Y.S., Nesterova, M., and Park, Y.G. *National Cancer Institute, Bethesda, MD 20892.*

Expression of the RIα subunit of cAMP-dependent protein kinase type I (PKA-I) is enhanced in human cancer cell lines and primary tumours. We have previously reported that a single injection treatment of RIα antisense triggered the suppression of RIα and PKA-I, and sustained inhibition of growth of human colon carcinoma in athymic mice. The growth inhibition accompanied the compensatory increase in RIIβ protein and PKA-IIβ (the RIIβ-containing PKA-II), and persistent down-regulation of PKA-I. We report here that these in vivo findings are consistent with observations made in cell culture, where we demonstrate that treatment of cancer cells with RIα antisense results in increased RIIβ protein without increases in the rate of RIIβ synthesis or the RIIβ mRNA level. Pulse-chase experiments revealed a 6-fold increase in the half-life of RIIβ protein. The half-life of RIIα protein did not change and RIα- and Cα- half-lives decreased slightly. An increase in the half-life of RIIβ protein was also found in LNCaP prostate cancer cells that demonstrate growth inhibition and RIα downregulation following RIα antisense treatment. This compensatory stabilization of RIIβ protein may represent a novel biological mechanism of RIα antisense that ensures depletion of protein kinase A type I and sustained inhibition of tumor growth.

#3402 CRE-transcription factor decoy oligonucleotide-induced growth inhibition of breast cancer cells involves p53-dependent signalling pathway. Lee, Y.N., Park, Y.G., and Cho-Chung, Y.S. *National Cancer Institute, Bethesda, MD 20892.*

The CRE, 5'-TGACGTCA-3' has been described as the consensus sequence for the cis-element that directs cAMP-induced gene transcription. The CRE-transcription factor complex is a pleiotropic activator that participates in the induction of a wide variety of cellular and viral genes. We previously reported that CRE-transcription factor decoy oligonucleotide induces the growth inhibition of many cancer cells *in vitro* and *in vivo*. The growth inhibition was accompanied by changes in cell morphology and apoptosis. To elucidate the molecular mechanism of the growth inhibition by the CRE-decoy oligonucleotide, we investigated the p53 signalling pathway. We found that CRE-decoy oligonucleotide treatment

#4554 Specific Inhibition of Human Heparanase Gene Expression in Human Cancer Cells by Adenoviral Antisense Construct. Futoshi Uno, Toshiyoshi Fujiwara, Masafumi Kataoka, Kou Katsuda, Takahiro Itoshima, Munenori Takaoka, Yoshio Naomoto, Noriko Uchiyama, Motowo Nakajima, and Toriaki Tanaka. *Department of Surgery 1, Shiga University Medical School, Otsu, Japan, Novartis Pharma K.K., Tsukuba, Japan, and Okayama University Medical School, Okayama, Japan.*

Heparan sulfate proteoglycans (HSPG) is one of the major components of the cell surface and extracellular matrix (ECM) and functions as a barrier against cationic molecules and macromolecules. The ability of heparan sulfate (HS) to interact with ECM macromlecules such as collagen, laminin and fibronectin, and with different attachment sites on plasma membrane indicates this proteoglycan is essential in self-assembly and insolubility of ECM components. Heparanase is endoglucuronidase capable of specifically degrading HS. Heparanase activity associated with metastatic potential of mouse lymphoma, fibrosarcoma and melanoma cell lines. Treatment of experimental animals with heparanase inhibitors considerably reduced the incidence of lung metastasis by melanoma and mammary adenocarcinoma cells. To specifically inhibit human heparanase gene expression in human cancer cells, we constructed antisense adenoviral vector containing a full-length human heparanase cDNA in the inverted direction that is driven by the potential CAG promoter (Ad-AS/hep). We also constructed an adenoviral vector expressing human heparanase gene (Ad-S/hep). RT-PCR and Western blot analysis detected increased expression of human heparanase in human esophageal and lung cancer cell lines (T.Tn and A549) following Ad-S/hep infection, which could be specifically inhibited by simultaneous infection with Ad-AS/hep in a dose-dependent manner. Infection with Ad-S/hep had no apparent effect on the growth of T.Tn cells, the invasive potential measured by the modified Boyden chamber assay, however, was significantly enhanced in T.Tn cells transduced with Ad-S/hep vector. Infection with Ad-AS/hep blocked this augmented invasive phenotype. It is important to establish the strategy to specifically inhibit the metastatic potential, which is a characteristic malignant phenotype of tumor cells. Locoregional administration of Ad-AS/hep vector might block tumor cell metastasis in vivo via downregulation of endogenous heparanase expression. Intrapleural injection of A549 human lung cancer cells induced massive pleural dissemination in BALB/c nu/nu mice 4weeks after tumor cell inoculation. Antitumor effect of intrapleural administration of Ad-AS/hep vector will be examined in this orthotopic pleural dissemination model.

#4555 *In Vivo* Studies of Antisense Oligonucleotides Targeted to the mRNA of Wilms' Tumor Gene In Tumor-Bearing Mice. Rosanne M. Orr, Jane Renshaw, Melanie R. Valenti, Lisa A. Brunton, Stewart Fleming, Edward V. Wancewicz, Mark J. Graham, Brett P. Monia, Kathy Pritchard-Jones, and Paul Workman. *Isis Pharmaceuticals Inc., Carlsbad, CA, The Institute of Cancer Research, Sutton, UK, and University of Edinburgh, Edinburgh, UK.*

The Wilms' tumor gene (*WT1*) is inappropriately expressed in the majority of acute leukemias. Previously, we have shown that the 2'-methoxyethyl chimeric oligonucleotides, ISIS 16601, targeting the 3'-UTR region of *WT1* mRNA, and ISIS 16609, targeting the exon 5 alternative splice site, have selectively reduced *WT1* mRNA and protein levels in K562 erythroleukemia cells *in vitro* (Proc. Am. Ass. Cancer Res. 41: 4798, 2000). Treatment of cells with both active oligonucleotides resulted in cytotoxicity. In this current study, we have established an *in vivo* model by injecting K562 cells, in presence of Matrigel®, into the right flank of athymic nude mice. When tumors reached 300-600mm^3, ISIS 16601, ISIS 16609 or ISIS 18389 (a control 4 base mismatched oligonucleotide to ISIS 16601) were administered by continuous s.c. infusion of 25mg/kg/day for 7 days by implantation of each oligonucleotide filled Alzet® micro-osmotic pump into the left flank of each mouse. Control tumor-bearing mice received saline s.c. infusions. This schedule was well tolerated and no hematological disturbances were observed. Since ISIS 16601 and ISIS 16609 have sequence homology to mouse *WT1*, bone marrow was removed for CD34$^+$ staining and kidneys for histology. Oligonucleotide treatment produced minimal effects on CD34$^+$ expressing immature bone marrow cells while kidney histology revealed some hematuria but no glomerular damage. Hematuria was not related to oligonucleotide sequence and was also observed following ISIS 18389 treatment. It is possible that this effect was caused by the binding of oligonucleotides to serum proteins since similar effects have been observed with cationized albumin. After 7 days, there was a 26% (P=0.06) and 48% (P=0.006) inhibition of tumor growth in ISIS 16601 and ISIS 16609 treated mice respectively compared with control tumors. Antitumor activity was accompanied by a 23% reduction in tumor WT1 protein levels (P=0.03) in ISIS 16609 treated mice, as measured by Western blotting, with intratumoral oligonucleotide concentrations in the micromolar range. These data suggest that further *in vivo* studies are warranted to assess the full chemotherapeutic potential of antisense oligonucleotides targeted to *WT1*.

#4556 Effects of DMT1 Antisense Oligodeoxynucleotide on Steroid Receptor Expression in Breast Cancer Cell Lines. Lan Yan, Sharyl J. Nass, and Nancy E. Davidson. *Johns Hopkins Oncology Center, Baltimore, MD.*

Estrogen and progesterone and their receptors (ERa and PR) play major roles in the development and function of the mammary gland as well as in breast cancer biology and treatment. However, 25-30% of human breast cancers lack ERa and PR expression and respond poorly to hormonal treatment. Absence of ERa and PR expression is associated with lack of transcript, methylation of the CpG islands in the promoter regions of these genes, and increased DMT activity and DMT1 protein. Treatment of ER-negative human breast cancer cell lines with a non-specific DMT inhibitor 5-azacytidine led to reactivation of functional ER protein. This study addresses the hypothesis that specific inhibition of DMT1 by antisense oligonucleotides (DMT1 ASO) is sufficient to reexpress ER and PR genes in ER-/PR- human breast cancer cell lines. MDA-MB-231 and Hs578t cells were transfected with 100 nM and 200 nM DMT1ASO respectively. Significant growth reduction was observed after 48 hr, and this inhibition was more obvious after 72 hr or 96 hr. DMT1 expression was blocked within 48 hr of exposure to DMT1 ASO as detected by Western blot whereas mutated DMT1 ASO had no effect. However, methylation specific PCR indicated that ER promoter CpG methylation was preserved and RT-PCR did not show reexpression of ER or PR mRNA after 48 hr. Prolonged exposure to DMT1 ASO for 72 and 96 hr was associated with DMT1 reexpression, possibly due to the short half-life of ASO. These results suggest that short-term inhibition of DMT1 is not sufficient to reactivate ER or PR expression in receptor-negative human breast cancer cells. The effects of more prolonged exposure, expression of other methylated target genes, and role of other members of the DMT family is under study. Supported by DAMD 17-98-1-8116 and NIHR01 CA78352.

#4557 Allele Specific Inhibition of Tumor Growth with Antisense Oligonucleotides in a Nude Mice Model with Fluorescent Tumors. Kees Fluiter, Anneloor L. ten Asbroek, Marcel Kool, Marjon van Groenigen, and Frank Baas. *Academic Medical Center, Amsterdam, Netherlands.*

Loss of heterozygosity (LOH) reduces many genes to hemizygosity in cancer cells and presents an absolute difference between normal and cancer cells. The regions of LOH are usually much larger than the tumor suppressor gene which is lost and are expected to contain multiple genes, some of which are essential for cell survival. Single nucleotide polymorphisms (SNP) are the most common type of genetic variation in man, often giving rise to two or more allelic forms of most genes. SNP's of essential genes that are frequently affected by LOH can be used as a target for a novel therapy against cancer cells with LOH. The SNP's can be targeted by antisense oligonucleotides (ODN) that will discriminate between two alleles. Allele specific inhibition of an essential gene will result in cell death in homozygous cancer cells while heterozygous normal cells will survive, provided that 50% of the gene activity is sufficient for cell survival. We have designed allele specific phosphorothioate ODN's against the large subunit of RNA polymerase II (POLR2A) located in close proximity to the tumor suppressor gene P53 on chromosome 17p13, a region that frequently shows LOH in cancer cells. To test the allele specific ODN's we developed a nude mouse model with tumor xenografts which express green fluorescent protein. The mouse was injected with two different tumor cell types subcutaneously, each homozygous for one of the two targeted alleles. The GFP expressing tumor cells showed degradation of the targeted POLR2A mRNA after transfection in vitro. We administered the allele specific antisense ODN's (5mg/kg body weight) continuously for 14 up to 28 days using osmotic minipumps implanted dorsally. Using laser light excitation and a CCD camera we could monitor the growth and angiogenesis of the tumor xenografts and the effects of the treatment with allele specific antisense ODN's in vivo. Using this model with fluorescent tumors we demonstrate allele specific inihibition of tumor growth with antisense oligonucleotides.

#4558 VEGF Inhibitor (AS-3m) Inhibits Growth of Selected Tumor Cells in Addition to Angiogenesis. Rizwan Masood, Jie Cai, Diane Lynne Smith, Sudhir Agrawal, and Parkash Singh Gill. *Hybridon Inc., Cambridge, MA, and University of Southern California, Keck School of Medicine, Los Angeles, CA.*

Various strategies for inhibiting vascular endothelial growth factor (VEGF) expression inhibit tumor angiogenesis. We have previously described the anti-tumor activity of a specific VEGF inhibitor, AS-3 (Masood et al., 1997, PNAS 94: 979-984). To extend these findings we studied 1) if VEGF receptor and VEGF expression occurred simultaneously in tumor cells; 2) whether cell lines co-expressing VEGF, VEGFR-2 and VEGFR-1 demonstrate autocrine growth factor activity; 3) whether cell lines with autocrine growth factor activity show alteration in the cell cycle in response to inhibition of VEGF expression and 4) whether VEGF inhibitor reduced tumor growth in murine xenograft models alone and in combination with chemotherapy. Expression of VEGF receptors and VEGF was detected by various methods, including mRNA and protein expression, in melanoma, ovarian carcinoma, and selected prostate and pancreatic carcinoma cell lines, but not in a myelomonocytic cell line (U937) which only expressed VEGFR1 or the transformed fibroblast cell line (T1) which did not express either receptor. AS-3 phosphorothioate oligonucleotide was modified to form a mixed backbone molecule in which the four deoxyribonucleosides at each end were replaced with 2' O-methyl ribonucleosides. The mixed backbone molecule (AS-3m) inhibited the proliferation of cell lines that co-expressed VEGF and both VEGFRs but minimal or no effect was observed in cell lines in which they were not co-expressed. A larger proportion of cells showed cell cycle arrest at G1 in AS-3m sensitive cell lines. Tumor xenografts of cell lines co-expressing VEGF, VEGFR-2 and VEGFR-1 showed marked reduction in the growth, with over 80% inhibition at a dose of 10mg/kg given daily beginning several days after the tumor implant. The levels of VEGF in the tumor tissue were significantly reduced and the effect was dose dependent. Furthermore, AS-3m had an additive effect when combined with chemotherapy. Tumor cell lines not expressing VEGFRs had markedly reduced inhibition of tumor growth. In conclusion, Many tumor cell lines co-express VEGF,

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Expression of DNA methyl-transferase (DMT) and the cell cycle in human breast cancer cells

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Estrogen receptor (ER)-negative breast cancer cells display extensive methylation of the ER gene CpG island and elevated DNA methyltransferase (DMT) expression compared to ER-positive cells. The present study demonstrates that DMT protein levels tightly correlate with S phase fraction in ER-positive cells, whereas ER-negative cells express DMT throughout the cell cycle. In addition, levels of $p21^{CIP1}$, which disrupts DMT binding to PCNA, are inversely correlated with DMT levels. Therefore increased DMT expression in ER-negative cells is not simply due to elevated S-phase fraction, but rather to more complex changes that allow cells to escape normal cell cycle-dependent controls on DMT expression. Because ER-negative breast tumors often have activated growth factor pathways, the impact of these pathways on DMT expression was examined in ER-positive cells. Stable transfection with fibroblast growth factors (FGFs) 1 and 4 led to increased DMT expression that could not be accounted for by a shift in S phase fraction. Elevated DMT protein expression in FGF-transfectants was accompanied by a significant decrease in p21, again suggesting a reciprocal relationship between these two proteins. However, acquisition of an estrogen-independent phenotype, even in conjunction with elevated DMT levels, was not sufficient to promote ER gene silencing via methylation. These results indicate that multiple steps are required for *de novo* methylation of the ER CpG island.

Keywords: breast cancer; estrogen receptor; DNA methylation; S phase; p21

Introduction

One major challenge in improving the treatment of breast cancer is understanding and overcoming resistance to endocrine therapy. Approximately one-third of human breast cancers lack estrogen receptor α (ER). These ER-negative tumors rarely respond to hormonal therapy with agents like the antiestrogen tamoxifen and are associated with shorter disease-free survival (McGuire, 1978; Samaan *et al.*, 1981; Early Breast Cancer Trialists Collaborative Group, 1998). Therefore, defining how and why tumors become ER-negative is a critical step for improving breast cancer therapeutic outcome. The molecular mechanisms underlying lack of estrogen receptor expression are poorly understood, but it has been hypothesized that ER-negative breast cancer cells may be derived from ER-positive cells that have acquired the ability to grow independently of estrogen and have lost expression of the gene.

Recent findings suggest that abnormal methylation of the ER gene CpG island may be important for silencing ER gene expression as some breast cancers progress to an aggressive, hormone insensitive phenotype (Ottaviano *et al.*, 1994; Lapidus *et al.*, 1996, 1998b; Ferguson *et al.*, 1995). CpG islands are cytosine–guanosine rich areas located in the 5′ regulatory region of some genes (Bird, 1986). Methylation of a CpG island results in transcriptional silencing of the associated gene, via a change in chromatin confirmation that inhibits transcription (Kass *et al.*, 1997). Cancer cells often display anomalous patterns of DNA methylation, with site specific hypermethylation in CpG island and hypomethylation of bulk genomic DNA (reviewed in Laird and Jaenisch, 1996; Counts and Goodman, 1995).

The CpG island in the promoter region of the ER gene is extensively methylated in established ER-negative breast cancer cell lines and primary tumors, but remains unmethylated in normal tissues and ER-positive breast cancer cell lines. Furthermore, treatment of the ER-negative cell line MDA-MB-231 with an inhibitor of DNA methylation results in demethylation of the ER CpG island and restores expression of a functional estrogen receptor (Ferguson *et al.*, 1995). However, it is not clear whether CpG island methylation initiates or maintains ER gene silencing, or whether aberrant methylation intrinsically accompanies the transition to an estrogen independent, ER-negative phenotype.

It is possible that abnormal methylation patterns result from overexpression of the enzyme that catalyzes cytosine methylation, DNA (cytosine-5) methyl-transferase (DMT). Expression of DMT is required for normal maintenance methylation, but changes in enzyme expression or activity may also promote *de novo* changes in a cell's methylation patterns. Increased expression of DMT is an early event in two experimental models of cancer (Belinsky *et al.*, 1996; Miyoshi *et al.*, 1993), and overexpression of DMT can promote a transformed phenotype in NIH3T3 cells (Wu *et al.*, 1993). In ER-negative breast cancer cell lines, DMT RNA and protein levels are significantly elevated compared to ER-positve cell lines (Ottaviano *et al.*, 1994; Ferguson *et al.*, 1997), but little is known about the regulation of DMT expression and activity in breast cancer. If deregula-

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tion of DMT is necessary to initiate or maintain aberrant methylation of the ER CpG island, then examining the regulation of DMT expression in breast cancer may greatly improve our understanding of breast cancer progression.

We therefore studied the expression of DMT in three panels of established human breast cancer cell lines that potentially represent different stages of breast cancer progression (estrogen-dependent ER-positive cell lines, estrogen-independent ER-positive lines, and ER-negative lines). We found that DMT expression was tightly correlated with S phase fraction in ER-positive cells, while ER-negative cells expressed DMT throughout the cell cycle. Thus, breast cancer cells may acquire characteristics that allow them to escape normal cell cycle-dependent regulatory controls on DMT expression during the process of tumor progression. Activation of peptide growth factor signaling pathways, which is common in breast cancers, led to increased DMT expression in ER-positive MCF7 cells without a concomitant change in S phase fraction. However, acquisition of an estrogen independent phenotype in MCF7 cells, even in conjunction with elevated DMT expression, was not inherently accompanied by aberrant methylation of the ER gene. Together, these data imply that multiple steps are required for *de novo* methylation of the ER CpG island.

Results

DMT levels correlate with S phase fraction in ER-positive, but not ER-negative cells

It had been previously demonstrated with six breast cancer cell lines that DMT mRNA, protein, and enzyme activity (Ottaviano *et al.*, 1994; Ferguson *et al.*, 1997), were elevated in ER-negative breast cancer cells compared to ER-positive cells. Because DMT protein is known to be expressed primarily during the S phase of the cell cycle in normal cells (Szyf *et al.*, 1985, 1991), we first determined whether the elevated DMT protein level in ER-negative cell lines was simply due to a large S phase fraction in those cells. Cell cycle distribution and DMT expression were examined during exponential growth in a larger panel of ten breast cancer cell lines (4 ER-positive and 6 ER-negative). For each cell line, nuclei for FACS analysis and total protein lysates for Western analysis were prepared concurrently. The results of the FACS analysis are shown in Figure 1. ER-positive cell lines generally had a higher G_1 fraction and a lower S phase fraction than the ER-negative lines. Western analysis confirmed that DMT protein levels were also lower in ER-positive than ER-negative cells (Figure 1). However, statistical analysis of these results showed a significant correlation between S phase fraction and DMT protein level in ER-positive cells ($r=0.99$, $P=0.009$, Figure 2a), but not ER-negative cells ($r=0.06$, $P=0.91$, Figure 2b), indicating that elevated DMT levels in ER-negative cells could not be explained solely by a change in S phase fraction. There was no significant correlation between DMT level and the fraction of cells in the other phases of the cell cycle (G_1 or G_2/M).

DMT and p21 are inversely correlated in breast cancer cells

A recent study reported that DMT can bind to PCNA (proliferating cell nuclear antigen), and that $p21^{CIP1}$ can disrupt the association between DMT and PCNA, possibly affecting the activity of these proteins (Chuang *et al.*, 1997). We therefore examined the levels of PCNA and p21 in the panel of ER-positive and -negative breast cancer cells (Figure 1). As shown in Figure 2c, DMT protein levels were inversely correlated with the level of p21 in breast cancer cells ($r=0.69$, $P=0.03$). Most ER negative cells expressed little or no p21, while expression levels were quite high in most ER-positive cell lines. PCNA expression did not vary greataly among the cell lines, and was not significantly correlated with DMT expression (Figure 1).

DMT antisense transfection leads to altered DMT/p21 ratio

The relative ratio of DMT protein to p21 protein was altered in MDA-231 cells stably transfected with a DMT antisense expression construct (Figure 3). Clones with decreased DMT expression also tended to have elevated p21 protein levels (mean relative DMT/p21 ratio of 1.7 compared to parental MDA-231 cells or vector-transfected controls (mean relative ratio of 42).

ER-negative cells express DMT protein in all phases of the cell cycle

To determine the cell cycle distribution of DMT-expressing cells, nuclei from exponentially growing cells were harvested, fixed, and analysed concurrently for PI staining and DMT expression. As shown in Figure 4 and Table 1, there was a striking difference between ER-positive and ER-negative cells. About 90% of cells in ER-negative lines expressed detectable amounts of DMT protein, whereas only 40% or less of ER-positive cells contained a

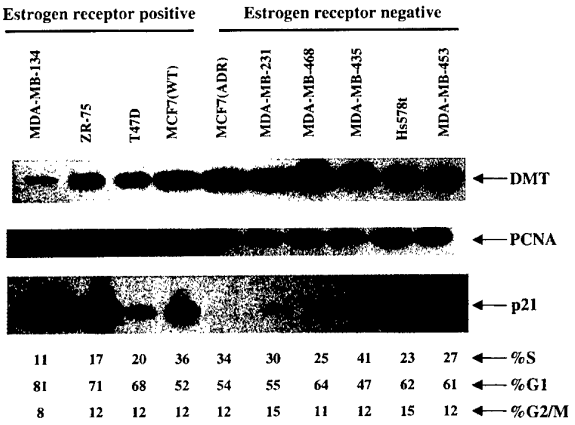

Figure 1 Expression of DMT, PCNA, and p21 protein in established human breast cancer cell lines. Whole cell lysates for Western analysis were prepared from a panel of 4 ER-positive and 6 ER-negative cell lines during exponential growth. Representative FACS analysis results for one experiment are shown beneath the Western blots

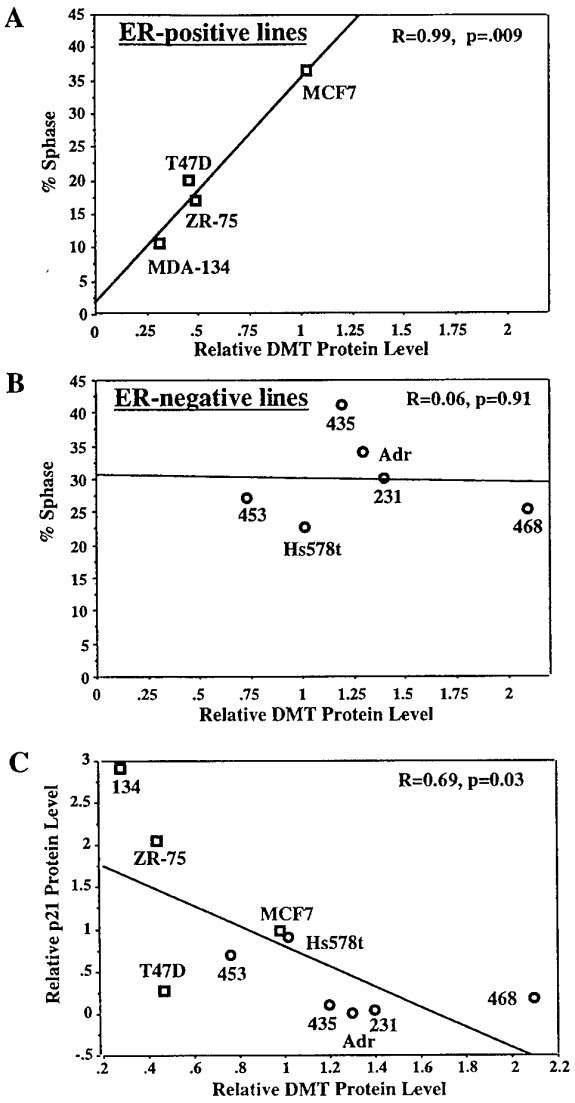

Figure 2 Relationship between relative DMT expression and other variables in human breast cancer cell lines. Representative results for one of three experiments are shown. (**a**) A tight correlation was observed between DMT protein levels and S phase fraction in ER-positive cells. (**b**) DMT protein level was not correlated with S phase fraction in ER-negative cells. (**c**) Relative p21 and DMT protein levels were inversely correlated ($P<0.05$). □, ER-positive lines; ○, ER-negative lines

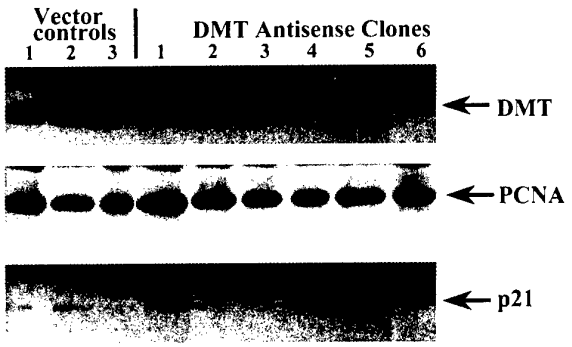

Figure 3 Expression of DMT, PCNA, and p21 protein in MDA-231 cells stably transfected with a human DMT antisense expression construct (pCMV-TMH). Vector controls were transfected by vector plasmids without insert DNA. DMT was not down-regulated in the first three antisense clones (1–3), and like the vector controls, p21 was barely detectable in these cell lines. In contrast, the three antisense clones (4–6) that did exhibit reduced DMT protein also showed up-regulation of p21 protein

Table 1 FACS analysis of human breast cancer cell lines stained for DMT protein and DNA content (cell cycle). $n=3$

Cell line	*% of total cells + for DMT*	*% of G_1 cells + for DMT*	*% of S cells + for DMT*	*% of G_2 M cells + for DMT*
ER-positive lines				
MDA-MB-134	20	17	51	42
T47d	30	21	67	63
ZR75	41	27	71	68
MCF7	42	23	70	62
ER-negative lines				
MCF7 ADR	98	97	99	98
MDA-MB-231	88	87	93	94
MDA-MB-468	91	88	97	96
MDA-MB-435	90	87	96	97
HS578t	88	80	96	97
MDA-MB-453	85	78	95	97

significant level of DMT. The most remarkable difference was observed in the G_1 phase of the cell cycle. In ER-positive lines, only about 20% of G_1 cells stained positive for DMT, whereas 80% or more of G_1 cells in ER-negative lines were DMT-positive. In addition, the S phase fraction of ER-positive lines consistently contained three times more cells with detectable DMT protein than the corresponding G_1 fraction, while ER-negative lines exhibited very little difference (10% or less) between the per cent of DMT-positive cells in the G_1 and S phases. Statistical analysis of the data (ANOVA) confirmed a significant difference ($P<0.01$) in the number of DMT-positive cells between ER-positive and ER-negative cell lines.

Estrogen independence does not lead to methylation of the ER CpG island in MCF7 cells

Many ER-negative breast tumors have up-regulated growth factor signaling pathways. To determine whether such pathways might play a role in the overexpression of DMT in breast cancer, MCF7 cells were treated with transforming growth factor α. Within 4 h, we observed a twofold increase in DMT protein levels without a concurrent increase in S phase fraction (not shown). Therefore, another panel of cell lines (Figure 5) was examined to determine whether progression of MCF7 cells to estrogen independence was accompanied by increased DMT expression and/or ER gene methylation.

The ER CpG island remained completely unmethylated in all of these transfected and selected MCF7 derivatives (Figure 6). This is in marked contrast to another MCF7 derivative, the MCF7(ADR) line, shown in Figure 3. The MCF7(ADR) cells developed an ER-negative phenotype with extensive methylation of the ER CpG island (Lapidus *et al*., 1998b) following selection for adriamycin resistance (Vickers *et al*., 1988).

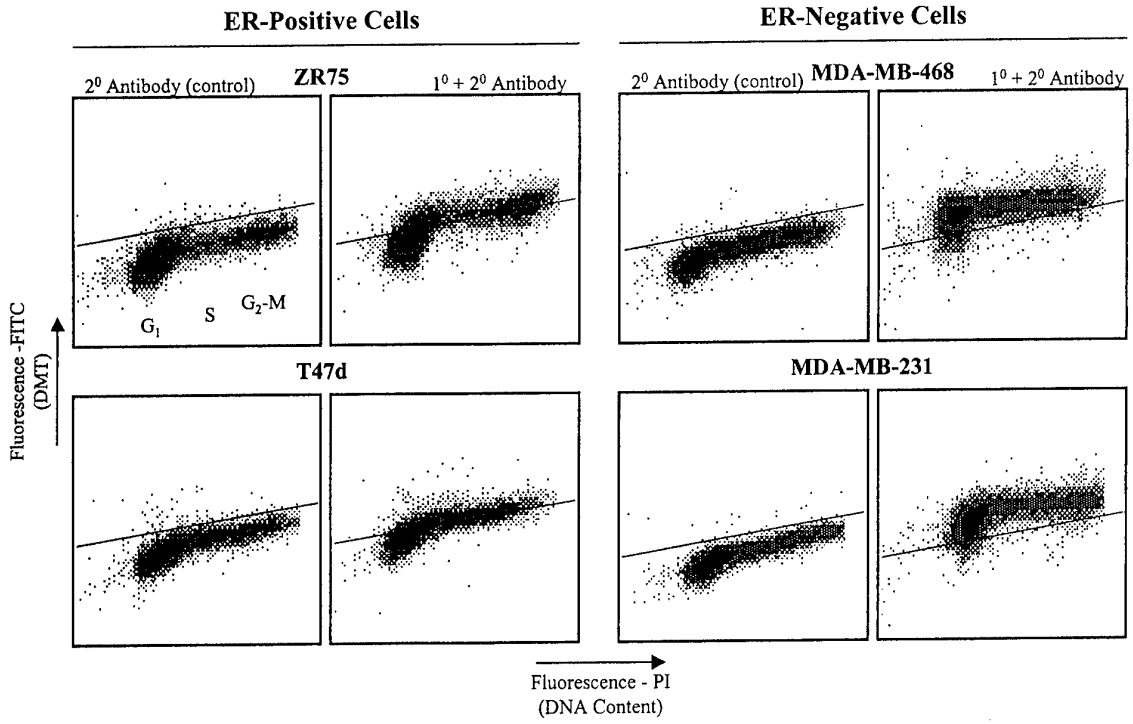

Figure 4 Concurrent analysis of cell cycle distribution and DMT expression. Fixed nuclei from exponentially growing cells were stained with PI as well as an anti-DMT primary antibody and an FITC-conjugated secondary antibody. ER-positive cell lines (ZR75, T47D) are shown on the left. ER-negative cell lines (MDA-231, MDA-468) are on the right. Control = PI + secondary antibody only

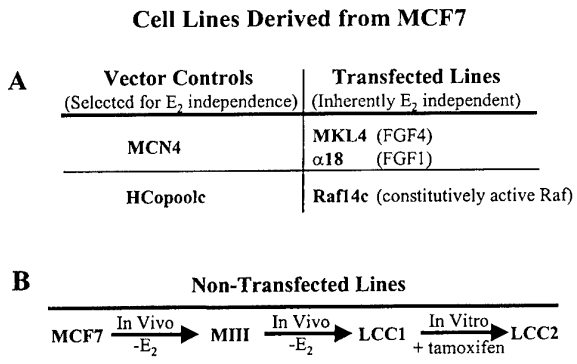

Figure 5 Derivation of the MCF7 sublines. With the exception of Raf14c, all sublines continue to express ER. (**a**) Cells transfected with FGF1, FGF4, or a constitutively active Raf are inherently estrogen-independent as a result of gene overexpression. Vector controls for these lines (MCN4 and HCopoolc) were selected for estrogen independence *in vitro* over a period of about 9 months. (**b**) The MIII and LCC1 lines were sequentially selected for estrogen-independent growth in nude mice, and LCC2 was derived from LCC1 by selection for resistance to tamoxifen *in vitro*

Selection for estrogen independence does not lead to deregulation of DMT

We next examined DMT protein levels and cell cycle distribution in these lines. Cell lines that had been selected *in vivo* for estrogen independence (MIII, LCC1, and LCC2) manifested DMT protein levels and S phase fractions similar to those observed for parental MCF7 cells (Figure 7). Vector control lines that had been selected for growth in CCS *in vitro* (MCN4c and HCopoolc) exhibited a comparatively low S phase fraction and expressed a relatively small amount of DMT. More importantly, all MCF7 variant lines selected for growth in the absence of estrogen *in vitro* or *in vivo* showed the same correlation between S phase and DMT level that was observed in the original panel of ER-positive cells (Figure 8a, $r = 0.858$, $P = 0.03$). The two regression lines are nearly parallel, with only a small shift to the left for the MCF7 variant lines, perhaps as a result of growth in CCS rather than FCS.

DMT protein level is elevated in transfected MCF7 cell lines

Constitutive expression of FGF1, FGF4, or Raf led to increased DMT protein levels compared to vector-transfected controls (Figures 7 and 8b). Transfection of MCF7 cells with FGF1 (α18) or FGF4 (MKL4) resulted in a fourfold increase in DMT levels that was not associated with a shift in S phase fraction (Figure 8b). In contrast, transfection with constitutively active Raf promoted a smaller increase in DMT level in conjunction with a shift in S phase fraction. In Figure 8b, the line showing the relationship between Raf14c and its vector control is parallel to the two regression lines in 8a.

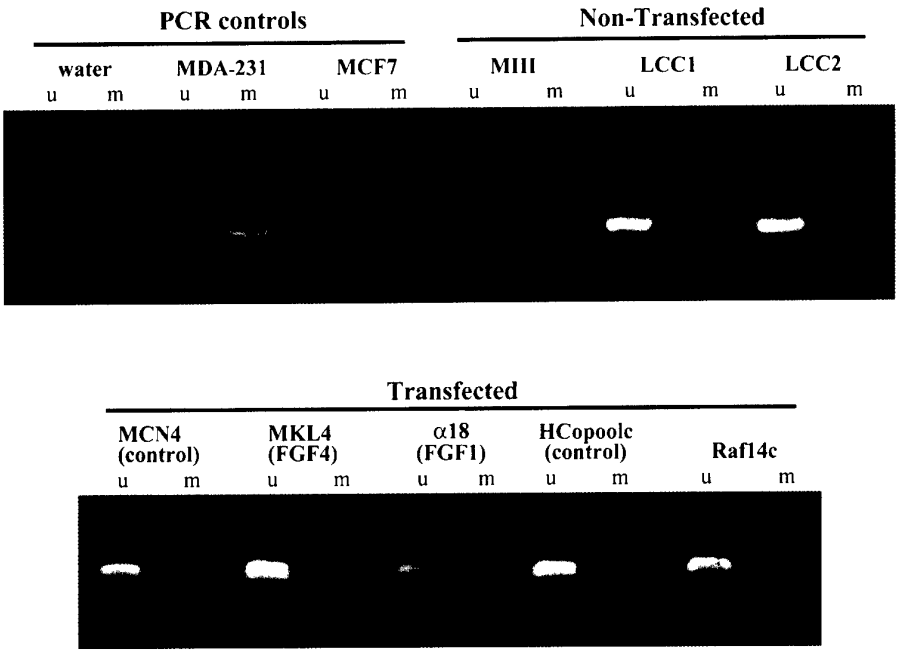

Figure 6 The ER CpG island remains unmethylated in estrogen independent lines derived from the ER-positive human breast cancer cell line MCF-7, as shown in Figure 5. Methylation specific PCR was used to assess the methylation status of multiple CpGs within the island. Representative results from one primer set are shown, since four different primer sets gave identical results. u = primers specific for unmethylated DNA, m = primers specific for methylated DNA. Water served as a negative control and DNA from MDA-MB-231 cells served as a positive control for the methylated reaction

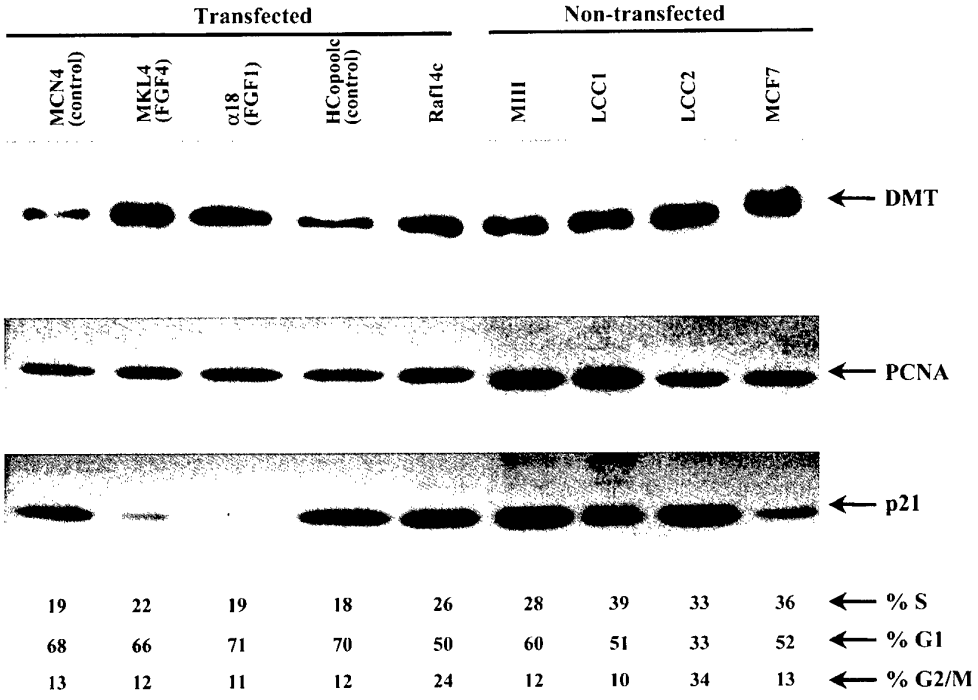

Figure 7 Expression of DMT, PCNA, and p21 protein in a panel of estrogen independent cell lines derived from MCF7 cells, as shown in Figure 5. Whole cell lysates for Western analysis were prepared from exponentially growing cells. Representative FACS analysis results for one experiment are shown beneath the Western blots. All cell lines were grown in CCS except untransfected MCF7 cells

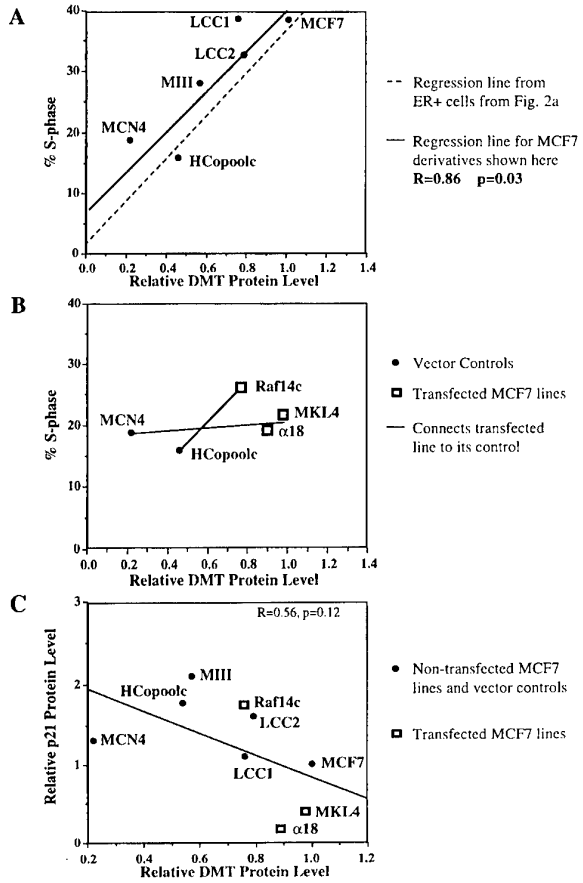

Figure 8 Relationship between relative DMT expression and other variables in MCF-7 variant cell lines. Representative results from one of three experiments are shown. (**a**) DMT levels were positively correlated with S phase fraction among untransfected or control vector-transfected cell lines, similar to results obtained for ER positive cell lines in Figure 2 (shown here as a dotted line). (**b**) Transfection of MCF7 cells with FGF1 (α18) or FGF4 (MKL4) resulted in a fourfold increase in DMT levels that was not associated with a shift in S phase fraction, while overexpression of constitutively active Raf promoted a parallel increase in DMT level (1.6 fold) and S phase fraction. Lines show the relationship between transfected lines and their specific controls. (**c**) A trend toward inverse correlation between relative p21 and DMT expression levels is noted ($P>0.05$)

p21 expression in MCF7 derived cell lines

A trend toward inverse correlation between DMT and p21 levels was noted among the MCF7 derivatives, but it did not achieve statistical significance (Figure 8c). However, the two FGF-transfected cell lines (α18 and MKL4) with significantly elevated DMT in the absence of increased S phase fraction, also showed a substantial decrease in p21 protein level (about sixfold).

Discussion

Defining the mechanism by which breast cancer cells develop an ER-negative phenotype is a crucial step for developing novel therapeutic strategies for hormone insensitive breast cancers. Hypermethylation of the ER gene CpG island is the only molecular change that has been consistently identified with the lack of ER gene expression in ER-negative breast tumors to date (Lapidus *et al.*, 1998a,b). However, it is not known how the ER gene becomes aberrantly methylated in these tumors. In fibroblasts, DMT overexpression can lead to *de novo* methylation of many promoter sequences (Vertino *et al.*, 1996). Several studies suggest that DMT expression is commonly elevated in cancer cells compared to normal cells of the same tissue type (Laird and Jaenisch, 1996), perhaps providing an explanation for the high frequency of abnormal CpG island methylation in cancer cells.

However, a recent study of colon tumors and matched normal colonic mucosa indicated that DMT expression in tumors is only modestly increased above that seen in normal colon tissue, and the increase in DMT expression was correlated with increased histone H4 expression, a measure of S phase-specific gene expression (Lee *et al.*, 1996). Those results were perhaps not surprising in light of the fact that DMT expression and activity are known to vary with the cell cycle (Szyf *et al.*, 1985, 1991). *In vivo*, DMT activity of non-proliferating tissues increases following a mitogenic stimulus with a time course coincident with entry into S phase (Szyf *et al.*, 1985). *In vitro*, DMT enzyme activity and mRNA levels in fibroblasts are both maximal during the S phase portion of the cell cycle (Szyf *et al.*, 1991).

Because DMT levels are elevated in ER-negative human breast cancer cell lines compared to ER-positive lines, it was important to determine whether this difference was due simply to altered cell cycle distributions or to a more complex phenomenon. In ER-positive cells, DMT expression was tightly correlated with S phase fraction. However, elevated DMT levels in ER-negative cells could not be explained solely by a change in cell cycle progression because DMT levels were not correlated with S phase in these cell lines. Thus, breast cancer cells may acquire characteristics that allow them to escape normal cell cycle-dependent regulatory controls on DMT expression during the process of tumor progression.

An increase in DMT expression in the absence of a concurrent increase in the percentage of cells in S phase could be achieved in one of two ways. First, DMT protein could simply be expressed at abnormally high levels during S phase. Alternatively, cell cycle dependent regulation of DMT protein expression could be lost or altered, resulting in inappropriate expression of the protein in other phases of the cell cycle. Our results indicate that the latter occurs in breast cancer. We demonstrated a striking difference between ER-positive and ER-negative cell lines in the cell cycle distribution of cells expressing detectable levels of DMT protein. DMT was detectable by FACS analysis during all phases of the cell cycle in ER-negative lines, with nearly all of the cells staining positive for DMT. In contrast, only 40% or less of ER-positive cells expressed detectable amounts of DMT, with the majority of G_1 phase cells negative for DMT, and the majority of S phase cells positive for DMT.

The normal association of DMT with the S phase of the cell cycle is not surprising, given the recent studies which reported that DNA methylation takes place concurrently with replication, perhaps via DMT binding to PCNA at the replication fork (Chuang *et*

al., 1997; Araujo *et al*., 1998). The cell cycle inhibitor $p21^{CIP1}$ can disrupt the association between DMT and PCNA, perhaps affecting the activity of the two proteins (Chuang *et al*., 1997). Furthermore, levels of DMT and p21 proteins were inversely related in normal and SV-40 transformed human fibroblasts. We therefore examined the levels of PCNA and p21 in the panel of ER-positive and -negative breast cancer cells and found that DMT protein levels were inversely correlated with the level of p21 in breast cancer cells. In contrast, PCNA expression did not vary greatly among the cell lines, and was not significantly correlated with DMT expression. It has been suggested (Chuang *et al*., 1997) that the transforming effect of DMT overexpression observed by others (Wu *et al*., 1993) may be due in part to the ability of DMT to compete with p21 for PCNA binding, thereby promoting the G_1-S phase transition. By binding to PCNA in place of p21, excess DMT could increase the level of active cyclin-dependent kinases, promoting Rb phosphorylation and thus progression through the cell cycle. If that hypothesis is correct, then the relatively high S phase fraction of ER-negative cells may be related to the high ratio of DMT to p21 in these cells. In this scenario, the increased DMT level in ER-negative cells could be a cause, rather than an effect, of a higher S phase fraction in those cells. However, the question still remains as to how DMT expression is deregulated in ER-negative breast cancer cells.

One potential mechanism for upregulation of DMT is altered transcriptional regulation. The mouse DMT gene has been reported to contain AP1 sites that can regulate its expression (Rouleau *et al*., 1995; Macleod *et al*., 1995). More recently, it was demonstrated that transformation of human fibroblasts by *fos*, a component of AP1, is dependent upon a *fos*-induced increase in DMT expression and activity (Bakin and Curran, 1999). Because many ER-negative tumors overexpress growth factor receptors whose signaling pathways include AP1 activation (Dickson and Lippman, 1995), we hypothesized that these pathways may play a role in the upregulation of DMT expression. In fact, the ER-negative breast cancer cell line with the highest level of DMT expression (MDA-MB-468) is known to overexpress the epidermal growth factor receptor (Filmus *et al*., 1985). Peptide growth factors stimulate several intracellular signaling pathways, but it is the Ras kinase pathway that leads directly to activation of the transcription factor AP1. Interestingly, the Hs578t cell line, which expressed DMT at a much higher level than would have been predicted by its relatively low S phase fraction, has a constitutively active mutant form of Ras (Kraus *et al*., 1984).

A second panel of cell lines, all of which were derived from the ER-positive MCF-7 line, was examined to further address these questions. Statistical analysis showed that DMT expression was still correlated with S phase in the MCF7-derived cell lines that had been selected for growth in the absence of estrogen, suggesting that aberrant expression of DMT does not necessarily accompany the progression to estrogen independence. However, constitutive overexpression of FGF1 or 4 led to a significant upregulation of DMT expression that could not be accounted for by a shift in S phase fraction. These results demonstrate that activation of growth factor signaling pathways can have an impact on the regulation of DMT expression. Overexpression of a constitutively active Raf kinase also led to increased DMT expression, but in this case, the change in expression was fully explained by a corresponding increase in S phase fraction. It is unclear why the results were different for the FGF and Raf transfectants, since all three proteins activate a Map kinase cascade. However, growth factors activate multiple intracellular pathways in addition to the Map kinase pathway. Perhaps activation of multiple pathways is necessary to disrupt the regulation of DMT protein expression in these cells. It is important to note that DMT protein levels can be regulated at the post-transcriptional level (Szyf *et al*., 1991), so even if DMT transcription is upregulated via Raf or AP1 activation, this may not be sufficient to elevate DMT protein levels. Additional post-transcriptional or post-translational regulatory mechanisms may supersede an increase in transcriptional activity.

The elevated DMT protein expression in FGF transfectants was accompanied by a significant decrease in p21, again suggesting a reciprocal relationship between these two proteins. Interestingly, we have also observed that the DMT/p21 ratio is significantly decreased in MDA-231 cells that are stably transfected with a DMT antisense expression vector. The fact that we have observed a similar phenomenon in two very different transfected model systems raises the question as to whether association with PCNA might affect the stability of these proteins. For example, because DMT and p21 compete for the same binding site on PCNA, an increase in DMT expression might promote dissociation of p21 from PCNA, perhaps making p21 more susceptible to ubiquitination and proteosome degradation (Maki and Howley, 1997). A decrease in DMT expression would then be expected to have the opposite effect on p21 stability.

In spite of our previous findings showing a connection between DMT expression and loss of ER expression, the elevated DMT expression in these ER-positive, estrogen independent cells was not sufficient to promote ER gene methylation and loss of ER gene expression. All cell lines derived from MCF-7 cells via transfection or selection grow in an estrogen-independent manner (Clarke *et al*., 1994; El-Ashry *et al*., 1997; Kern *et al*., 1994). However, all but one (Raf14c) continue to express ER protein. Furthermore, the ER CpG island remained completely unmethylated in all of these cell lines, including Raf14c. Thus, acquisition of an estrogen-independent phenotype, even in conjunction with loss of ER expression in the Raf14c line, was not sufficient to promote ER gene silencing via CpG island methylation. These results are not due to an inherent inability of the MCF7 cells to methylate the ER gene. We know that MCF7 cells can acquire the capability to methylate the ER gene and silence its transcription because the MCF7ADR line, which was selected for resistance to adriamycin, is ER negative and shows deregulated DMT expression and extensive ER CpG island methylation (Lapidus *et al*., 1998b).

These results suggest that ER gene transcription must first be down-regulated by another mechanism, perhaps via altered availability of a critical transcription factor, prior to DNA methylation. In this scenario, aberrant CpG island methylation may act

to consolidate and strengthen transcriptional silencing of the associated gene. Although it has not been definitively demonstrated that ER-positive cells progress to ER-negative breast tumors, we propose that such a transition would require the tumor cells to pass through at least three stages during the progression from an estrogen dependent phenotype, to a stable, ER-negative phenotype. These stages would include acquisition of estrogen independence, loss of ER transcription, and finally ER CpG island methylation. It is not yet clear whether aberrant DMT expression precedes ER gene methylation, but our results with the Raf-transfected cell line suggest that loss of ER expression alone is not sufficient to trigger ER gene methylation. Although elevated DMT protein expression in the FGF model system was not sufficient to induce ER CpG island methylation, deregulation of DMT expression or activity may still be a prerequisite for *de novo* methylation of the ER gene in breast cancer cells. Alternatively, an increase in DMT expression may be necessary to maintain the abnormal methylation pattern after the gene has been silenced.

Materials and methods

Cell lines

Four ER-positive (MCF-7/WT, ZR-75-1, T47D, and MDA-MB-134) and six ER negative (MCF-7/Adr, Hs578t, MDA-MB-231, MDA-MB-435, MDA-MR-453, and MDA-MB-468) human breast cancer cell lines were acquired and routinely maintained as previously described (Ottaviano *et al*., 1994). Eight additional cell lines derived from the ER-positive MCF-7 line were also examined (Figure 5). Raf14c (overexpressing a constitutively active form of Raf) and its vector control, HCopoolc, as well as MKL4 and α18 cells (which overexpress FGF4 and FGF1 respectively) and their vector control, MCN4 were provided by Dr Dorraya El-Ashry and Dr Francis Kern of the Lombardi Cancer Center and Southern Research Institute, respectively (El-Ashry *et al*., 1997; Kern *et al*., 1994). These transfected lines have acquired estrogen independent growth as a result of their specific gene overexpression and are grown continuously in the absence of estrogen. The MCN4 and HCopoolc cell lines were selected *in vitro* for estrogen independent growth over a period of about nine months (El-Ashry *et al*., 1997; Kern *et al*., 1994). MCF-7/MIII, MCF-7/LCCI, and MCF-7/LCC2 (Clarke *et al*., 1994) were a gift from Dr Robert Clarke (Lombardi Cancer Center, Washington DC, USA). The MIII and LCC1 lines sequentially selected for estrogen-independent growth in nude mice, and LCC2 was derived from LCC1 by selection for resistance to tamoxifen *in vitro*. All estrogen independent MCF7 derivatives were grown in IMEM without phenol red supplemented with 10% serum that had been charcoal-stripped to remove all steroid hormones (CCS, HyClone Laboratories, Logan, Utah, USA). With the exception of Raf14c, all MCF7 derivatives continue to express ER protein.

FACS analysis

Cells were plated in 100-mm tissue culture dishes concurrently for cell cycle analysis by FACS and for Western analysis. Two days later, exponentially growing cells were harvested at about 70% confluence. Nuclei were isolated and stained with propidium ioidide (PI) for cell cycle analysis according to the method of Vindelov *et al*. (1983).

To determine the cell cycle distribution of cells expressing DMT, nuclei were fixed and stained by immunofluorescence for DMT along with PI. Growing cells were harvested by trypsinization and then swelled in HSSE buffer (20 mM HEPES, pH 7.0, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) on ice for 20 min. Cells were lysed in a dounce homogenizer and nuclei were pelleted by centrifugation. The nuclei were resuspended in 0.5 M sucrose/HSSE, layered on a cushion of 1.5 M sucrose/HSSE, and centrifuged for 20 min at 13 000 *g*. Isolated nuclei were then fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) for 10 min on ice, washed with PBS, and stored at −70° C prior to FACS analysis.

The nuclei were resuspended in permeabilization buffer (PBS with 4% goat serum and 0.1% Triton X-100), washed with PBS, and incubated with 1 μg/ml primary DMT antibody in PBS with 1% BSA for 2 h at room temperature (RT). Following washes in PBS/1% BSA, the nuclei were incubated for 1 h at RT with an FITC-conjugated anti-rabbit secondary antibody (Sigma) diluted 1/500 in PBS/1% BSA. Nuclei were washed again in PBS and then stained with PI as above. FACS analysis was repeated at least three times for all cell lines examined.

Preparation and characterization of anti-DMT antiserum

With the help of Research Genetics, Inc., rabbit polyclonal antiserum was raised against a peptide derived from the N-terminal region of DMT (NH_3-MADANSPPKPLSKPRT-PRRSCOOH). The peptide was conjugated to KLH and used for rabbit immunization and boosting. The resultant antiserum against the peptide recognized a single polypeptide at the expected molecular weight on an immunoblot of proteins derived from a variety of human cell lines, and depleted DNA methyltransferase activity from nuclear extracts (not shown). Affinity purified antibody was used for FACS and Western analysis.

Western analysis

Cells were plated as described above. Two days later, total cell lysates were prepared. Cells were washed with cold PBS and then scraped into cold lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). After a 10 min incubation on ice, lysates were spun for 10 min in a cold microcentrifuge to remove cellular debris and were frozen at −70°C. Proteins were boiled and separated by SDS–PAGE and then transferred to nitrocellulose membranes. For DMT analysis, 75 μg of protein from each sample were separated on 6.5% polyacrylamide gels. For PCNA and p21, 25 μg of protein were run on 14% gels. Blots were blocked in 7% BSA in Tris-buffered saline with Tween-20 (TBST; 10 mM Tris, pH 7.4, 150 mM NaCl, 1% Tween-20) for 1 h at room temperature and then incubated in TBST with 1% BSA and the following antibodies (1 μg/ml): DMT (polyclonal), PCNA (monoclonal, Oncogene), or p21 (monoclonal, Oncogene). Proteins were visualized with an HRP-linked second antibody (1/5000 in TBST with 1% BSA) and a chemiluminescent detection system (Pierce, Rockford, IL, USA). India ink staining of the membranes demonstrated equal loading and transfer of the samples.

Densitometry and statistical analysis

Western band intensities were quantitated with IPLab Gel 1.5 (Scanalytics, Fairfax, VA, Canada). Relative protein levels were calculated relative to the values obtained for the parental MCF-7 line that was assigned an arbitrary value of 1. Correlations between relative DMT protein levels and the other variables examined (% cells in S, G_1, or G_2/M phase, and relative p21 or PCNA protein levels) were calculated with Statview 4.02. Significance was set at $P<0.05$.

DMT antisense transfection

MDA-231 cells were stably transfected with pCMV-TMH, a human antisense DMT constitutive expression plasmid provided by Dr Paula Vertino at the Emory University School of Medicine (Vertino *et al.*, 1996). Clones were selected in 1 mM G418 and screened for altered DMT expression by Western analysis.

Methylation specific PCR (MSP)

ER CpG island MSP was performed as previously described (Lapidus *et al.*, 1998b). Four primer sets that span the ER CpG island were used for the analysis (primers sets 1, 3, 4 and 5 from Lapidus *et al.*, 1998b). Controls lacking DNA were included with each set of PCR reactions. Ten μl of total reaction was run on a 3% TBE/agarose gel, stained with ethidium bromide, and visualized by UV light. Eagle Eye technology (Stratagene) and standard graphic software packages were utilized to prepare figures.

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Advances in Brief

Aberrant Methylation of the Estrogen Receptor and E-Cadherin 5′ CpG Islands Increases with Malignant Progression in Human Breast Cancer[1]

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Abstract

Loss of expression for both the *estrogen receptor-α* and *E-cadherin* genes has been linked to disease progression in human ductal breast carcinomas and has been associated with aberrant 5′ CpG island methylation. To assess when, during malignant progression, such methylation begins and whether such methylation increases with advancing disease, we have surveyed 111 ductal carcinomas of the breast for aberrant methylation of the estrogen receptor- α and E-cadherin 5′ CpG islands. Hypermethylation of either CpG island was evident prior to invasion in ~30% of ductal carcinoma *in situ* lesions and increased significantly to nearly 60% in metastatic lesions. Coincident methylation of both CpG islands also increased significantly from ~20% in ductal carcinoma *in situ* to nearly 50% in metastatic lesions. Furthermore, in all cases, the pattern of methylation displayed substantial heterogeneity, reflecting the well-established, heterogeneous loss of expression for these genes in ductal carcinomas of the breast.

Introduction

Human breast carcinomas most frequently evolve from the epithelial lining of the terminal mammary ducts as DCIS[3] that may progressively become invasive and ultimately metastatic (1). The transformation of normal mammary epithelial cells into a carcinoma and the subsequent progression to invasion and metastasis involve the accumulation of numerous genetic "hits," including the activation or amplification of dominant oncogenes and the deletion or inactivating mutation of key tumor suppressor genes (2). It has recently become evident that tumor suppressor genes may also be transcriptionally silenced in association with aberrant promoter-region CpG island methylation (3, 4).

The *ERα* gene and the *E-cad* gene have been implicated frequently in the initiation and/or progression of human breast cancer. Loss of expression of either gene has been associated with poorly differentiated tumors and poorer prognosis (5–10). Furthermore, several studies have reported an association between E-cad and ER expression in breast tumors (7, 9, 10). In the case of E-cad, classical mutations and deletions may play a role in loss of gene expression (11, 12). However, loss of E-cad expression, as well as loss of ER expression, has also been associated with aberrant 5′ CpG island methylation in breast cancer cell lines and primary human breast tumors (13–18). It is currently unclear when, during malignant progression of ductal breast carcinoma, aberrant methylation of these CpG islands begins and whether the incidence of such methylation tracks with advancing disease for either or both genes. Therefore, we have evaluated a total of 111 ductal breast carcinomas for the incidence of CpG island methylation for these two key suppressor genes in *in situ*, invasive, and metastatic lesions. Our results indicate that the aberrant methylation of either CpG island begins before invasion and increases with metastatic progression. Coincident methylation of both CpG islands also increases with progression, suggesting that the malignant progression of ductal breast carcinoma involves the accumulation of multiple epigenetic "hits."

Materials and Methods

Tissue Samples. A total of 111 human breast tumor samples identified as DCIS, IDC, and LA/MDC were obtained from the Department of Pathology at Johns Hopkins University School of Medicine and from the Department of Pathology at Vanderbilt University Hospital. Seventy-five % of the LA/MDC samples were derived from lymph nodes, whereas the remaining 25% consisted of samples from a variety of sites including the chest wall, bone, and lung. Two cases of recurrent breast cancer after lumpectomy were also included. In the case of DCIS, samples were carefully microdissected prior to DNA isolation to avoid sample contamination with other cells. A portion of these tumors had been analyzed previously for E-cad methylation (18). The preliminary results of that study prompted us to expand the tumor sample pool and to include analysis of a second gene (ER). None of the results for ER methylation in this tumor set have been reported previously.

Cell Lines. Two human breast cancer cell lines were used as controls for methylation assays. MCF-7 cells express both ER and E-cad, and the CpG islands of both genes are unmethylated in this cell line. The MDA-MB-231 cell line exhibits extensive methylation of the *ER* and *E-cad* gene CpG islands, and the cells lack expression of the two genes at both the mRNA and protein level (13, 14, 16, 17). The cell lines were routinely maintained as described previously (13).

DNA Isolation. DNA was isolated from the tissues and cell lines as described previously (14, 16). DNA samples were labeled with a coded identification number so that MSP analysis could be performed and analyzed without knowledge of the sample origin.

MSP. ER and E-cad 5′ CpG island MSP was performed on sodium bisulfite-treated DNA as described previously (15, 17). The ER primers (primer set #5; Ref. 15) target a region of the gene about 400 bp downstream from the transcription start site near a *Not*I site. MSP primers spanning the transcription start site of E-cad were described previously as Island 3 (17). Earlier studies showed that methylation in the regions targeted by these primer sets correlated best with loss of gene expression (15, 17). A fraction of the tumor samples in the current study were also analyzed with additional MSP primer sets for the two genes to verify the density of CpG island methylation in these tumors. For many samples, the methylation status of ER and E-cad was assessed concurrently by including primers for both genes in the same reaction (termed duplex PCR).

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[3] The abbreviations used are: DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; E-cad, E-cadherin; MSP, methylation-specific PCR; IDC, invasive ductal carcinoma; MDC, metastatic ductal carcinoma; LA, locally advanced.

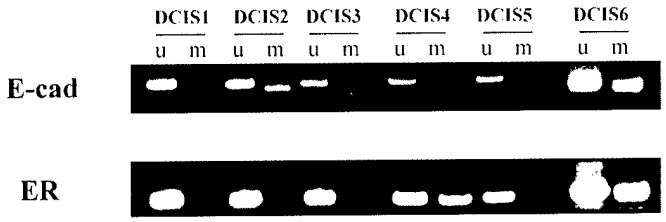

Fig. 1. MSP analysis of the E-cad and ER CpG islands in human breast cancers (DCIS). MSP was used to assess the methylation status of each CpG island. Representative results from six DCIS lesions are shown. The two genes were analyzed concurrently by performing duplex PCR reactions that contained primers for both islands. *u*, primers specific for unmethylated DNA; *m*, primers specific for methylated DNA.

Statistical Analysis. Any tumor sample that reliably yielded a PCR product in the methylated reaction visible by ethidium bromide staining was considered positive for CpG methylation. The Mantel-Haenszel χ^2 test for trend was applied to 3-by-2 tables of tumor type *versus* methylation (yes/no) to assess the change in percentage of methylation with increasing tumor progression. Then each pair of tumor types was compared using logistic regression. Significance was set at $P < 0.05$.

Results and Discussion

The ER and the homotypic cell:cell adhesion molecule, E-cad, both play a role in maintaining the normal differentiated state of the mammary gland epithelium (6, 19). Loss of the ER during breast cancer progression is associated with poorer histological differentiation, higher growth fraction, and poorer clinical outcome and may represent a key mechanism facilitating hormone resistance (5, 20). Similarly, loss of E-cad expression has been repeatedly associated with loss of differentiation, increased invasive and metastatic potential, and decreased patient survival (6, 9, 11, 21). The transcriptional silencing of both ER and E-cad in human breast cancer has been associated with aberrant promoter-region CpG island hypermethylation. In addition, treatment of human breast cancer cell lines lacking ER and/or E-cad with DNA methyltransferase inhibitor (5-deoxyazacytidine) elicits CpG island demethylation and re-expression of E-cad and ER protein, thereby indicating that aberrant methylation of these CpG islands plays a substantial role in suppressing transcription of these two key suppressor genes in breast cancer cells (16, 22).

Because expression of both ER and E-cad is lost in association with aberrant 5′ CpG island methylation during breast tumorigenesis, we sought to define the stage of breast tumor progression at which the hypermethylation of these two CpG islands begins and whether such methylation tracks with advancing disease. We analyzed a total of 111 ductal breast carcinomas comprised of *in situ* lesions (DCIS), invasive, and metastatic cancers by MSP (23).

The Incidence of CpG Island Methylation Increases with Tumor Progression. MSP has been used previously to detect aberrant DNA methylation of several genes, including *ER* and *E-cad,* in human cancers (15, 17, 22). Neither gene is methylated in normal breast epithelia (13–17). However, methylation of the two CpG islands was evident in all tumor stages and showed remarkably similar increases during progression from DCIS to metastatic tumors. Methylation of the *ER* gene was evident in 34% (12 of 35) of DCIS lesions, whereas E-cad methylation was evident in 31% (11 of 35). Coincident methylation was present in only 21% of these DCIS lesions. (Fig. 1 and summarized in Table 1). In invasive and metastatic ductal carcinomas (IDC or MDC), the incidence of methylation markedly increased relative to the DCIS lesions. Twenty-five of 48 (52%) IDC samples showed methylation of the ER or E-cad 5′ CpG island (Fig. 2; Table 1). Of these 48 samples, 18 (38%) showed distinct, coincident methylation of both CpG islands. Of the locally advanced and metastatic tumor samples, nearly 60% exhibited methylation for each of the CpG islands (Fig. 2; Table 1), whereas coincident methylation of both CpG islands was apparent in 50% (14 of 28) of these samples.

These data indicate that the epigenetic inactivation of either gene may occur early, prior to invasion, but increases as cells acquire invasiveness and metastatic potential. The Mantel-Haenzael χ^2 test for trend demonstrated that the trend toward increased methylation during progression was statistically significant for each gene ($P < 0.05$; Table 1). Furthermore, pair-wise comparison of the three tumor stages demonstrated that the incidence of methylation in metastatic tumors was significantly higher than in DCIS for both ER (odds ratio, 2.96; $P = 0.039$) and E-cad (odds ratio, 3.37; $P = 0.022$). The incidence of methylation in IDC samples was not statistically different from the other two categories, however.

The trend toward increasing coincident methylation of the two genes during progression was also statistically significant ($P = 0.013$;

Table 1 *Incidence of CpG island methylation for ER and E-cad genes in human breast tumors*

Tumor type	% ER methylation	% E-cad methylation	% ER and E-cad methylation	% ER or E-cad methylation
All	49% (54/111)	48% (53/111	35% (39/111)	61% (68/111)
DCIS	34% (12/35)	31% (11/35)	21% (7/35)	46% (16/35)
IDC	52% (25/48)	52% (25/48)	38% (18/48)	67% (32/48)
LA/MDC	61% (17/28)	61% (17/28)	50% (14/28)	71% (20/28)
P (trend) M-H[a] χ^2	0.034	0.019	0.013	0.032

[a] M-H, Mantel-Haenszel.

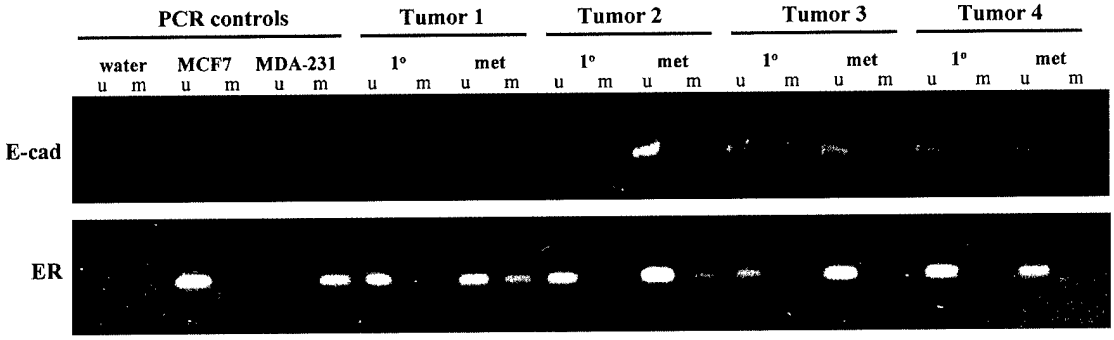

Fig. 2. MSP analysis of the E-cad and ER CpG islands in human breast cancers (IDC and MDC). Representative results from four primary (*1°*)-metastatic (*met*) pairs are shown. MSP reactions for E-cad and ER were run and analyzed separately. Metastatic sites were as follows: *1*, bone; *2*, chest wall; *3*, axillary lymph node; *4*, supraclavicular lymph node. *u*, primers specific for unmethylated DNA; *m*, primers specific for methylated DNA. Water served as a negative control, and DNA from MCF-7 and MDA-MB-231 cells served as positive controls for the unmethylated and methylated reactions, respectively.

Table 1). Thus, the frequency of coincident methylation of both CpG islands increases with advancing disease, suggesting that malignant progression of ductal breast carcinoma involves the accumulation of multiple epigenetic "hits." However, it is important to note that the similarity in the trends for ER and E-cad methylation was not attributable to complete coincidence of methylation for the two genes. At every stage of progression, the rate of coincident methylation was lower than the incidence of methylation for each individual gene (Table 1). Overall, ~25% of the samples analyzed showed methylation of either ER or E-cad, but not both. These results imply that aberrant methylation of these CpG islands does not simply reflect a generalized increase in CpG island methylation but may reflect a more specific selection process targeting key suppressor genes.

CpG Island Methylation Is Heterogeneous in Breast Tumors. In all samples harboring methylation, unmethylated alleles were invariably also evident (Figs. 1 and 2). For the IDC and LA/MDC samples, which were not microdissected, these unmethylated alleles may reflect the contribution from normal cells in the sample. Alternatively, these alleles may be derived from cancer cells that harbored only unmethylated copies of the E-cad and ER CpG islands. However, this same heterogeneous pattern was evident in the methylated DCIS samples, which were carefully microdissected, suggesting that methylation of these CpG islands in these tumors is heterogeneous. Interestingly, expression studies have routinely revealed that the loss of both E-cad and ER exhibits distinct heterogeneity in ductal breast carcinomas (6, 9, 10, 24). In addition our earlier studies have demonstrated that heterogeneity of both ER (15) and E-cad (18) methylation is associated with heterogeneity of protein expression. Limitations in our ability to recover the tissue specimens associated with these DNA samples (especially those derived from *in situ* lesions) precluded a simultaneous evaluation in this study. However, it seems likely that the heterogeneous patterns of CpG island methylation parallel the heterogeneous loss of E-cad and ER expression in these tumors.

In summary, these data indicate that the malignant progression of human ductal breast carcinomas involves a heterogeneous pattern of methylation for both the ER and E-cad 5′ CpG islands that begins prior to the acquisition of invasiveness and increases for each CpG island with advancing disease. In the case of E-cad, these results are particularly striking because loss of E-cad expression is generally associated with the acquisition of invasive or metastatic potential rather than the earlier stages of tumorigenesis. Finally, the increase in the coincident methylation of both CpG islands suggests that malignant progression of human breast cancer involves not only the well-documented accumulation of genetic "hits" but also an accumulation of epigenetic "hits" that contribute to the diminished expression of key tumor suppressor genes like *ER* and *E-cad*.

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Advances in Brief

Transcriptional Activation of Estrogen Receptor α in Human Breast Cancer Cells by Histone Deacetylase Inhibition[1]

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Abstract

Recent findings have established a connection between DNA methylation and transcriptionally inactive chromatin characterized by deacetylated histones. Because the absence of *estrogen receptor α* (*ERα*) gene expression has been associated with aberrant methylation of its CpG island in a significant fraction of breast cancers, we tested whether histone deacetylase activity contributes to the transcriptional inactivation of the methylated *ER* gene in a panel of ER-negative human breast cancer cells. Treatment of these cells with trichostatin A, a specific histone deacetylase inhibitor, led to dose- and time-dependent re-expression of ER mRNA as detected by reverse transcription-PCR without alteration in *ERα* CpG island methylation. Trichostatin A-induced ER re-expression was associated with increased sensitivity to DNase I at the *ER* locus in MDA-MB-231 cells. These data implicate inactive chromatin mediated by histone deacetylation as a critical component of *ER* gene silencing in human breast cancer cells. Therefore, histone deacetylation may be a potential target for therapeutic intervention in the treatment of a subset of ER-negative breast cancers.

Introduction

Interaction between 17β-estradiol and ERα[3] plays an important role in breast carcinogenesis and breast cancer treatment. That estrogen stimulates the growth of certain breast cancers is well established, and hormonal therapy via estrogen depletion or antiestrogen administration is widely used to block the action of estrogen in women with breast cancer. However, patients whose breast cancers lack ER seldom respond to endocrine therapy; therefore, a potential mechanism for hormone resistance is *de novo* or acquired loss of *ER* gene expression at the transcriptional level during disease progression (1, 2).

One possible mechanism for loss of ER in ER-negative breast cancers is cytosine methylation of the *ER* CpG island in the 5′ regulatory region of the gene (3). Indeed the *ER* gene CpG island is extensively methylated in ER-negative breast cancer cells, and ~50% of unselected primary breast tumors but remains unmethylated in normal breast tissue and many ER-positive tumors and ER-positive cancer cell lines (4, 5). The functional importance of this finding is demonstrated by the fact that treatment of ER-negative human breast cancer cells with the demethylating agent, 5-aza-dC, led to reactivation of expression of ER mRNA and functional ER protein (6). Recent studies indicate that silencing of a gene by methylation involves the generation of an inactive chromatin structure characterized by deacetylated histones. An abundant chromosomal methyl CpG-binding protein, MeCP2, was the first protein identified to link methylated DNA and a HDAC-containing transcriptionally repressive complex for gene silencing. Subsequently, several MBD proteins have been identified that, similar to MeCP2, couple methylated DNA to HDAC (7, 8). More recently, the well-known maintenance methyltransferase, Dnmt1, was found to interact physically with HDAC through its N terminus, thereby forming a transcriptionally inactive chromatin structure that represses transcription (9, 10). All of these findings demonstrate the important role of HDAC in transcriptional regulation. The HDACs deacetylate lysine groups of histones H3 and H4, allowing ionic interactions between positively charged lysines and negatively charged DNA, which result in a more compact nucleosome structure that limits transcription. The availability of specific HDAC inhibitors such as TSA (11) permits the study of the role of HDAC in silencing a variety of tissue-specific methylated genes (7, 12).

Here, we have tested whether loss of ER expression in some breast cancers is associated with transcriptional repression through HDAC activity on the methylated *ER* gene. Our data demonstrate that specific HDAC inhibition via TSA treatment can reactivate ER transcription in the presence of the methylated DNA. The activated gene transcription is associated with increased sensitivity of the *ER* promoter to DNase I treatment. These data suggest that inactive chromatin mediated by HDAC is critical to *ER* gene silencing.

Materials and Methods

Cell Lines, Reagents, and TSA Treatment. Human breast cancer cells (Hs578t, MCF-7/WT, MCF-7/Adr^R, T-47D, and MDA-MB-231) were grown in DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine. TSA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), dissolved in absolute ethanol at a stock concentration of 1 mg/ml (3.30 mM), and stored at −20°C. DNase I was purchased from Pharmacia Biotech (Pharmacia Biotech, NJ). Cells (MDA-MB-231, Hs578t, T-47D, or MCF-7/Adr^R) were seeded at a density of 6×10^5 cells/100-mm tissue culture dish (8×10^3 cells/cm^2). After 24 h of incubation, the culture medium was changed to different concentrations of TSA- or vehicle (ethanol)-containing medium. Either total cellular RNA or genomic DNA was isolated after 0, 24, 48, or 72 h of TSA exposure.

RNA Isolation and RT-PCR Analysis of ER Expression. Total cellular RNA was isolated from cell lines with TRIzol reagent according to the recommendations of the supplier (Life Technologies, Inc., Rockville, MD). RNA (3 μg) was reversibly transcribed by Moloney murine leukemia virus reverse transcriptase (Life Technologies) using OligoDT_{15} primer (Promega Corp., Madison, WI) in a final volume of 50 μl. Four % of synthesized cDNA (2 μl, derived from 150 ng of initial RNA) was used for PCR amplification of ER and the constitutively expressed housekeeping gene *β-actin* (13). Specific sense and antisense PCR primers used for the amplifications across the seventh

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[3] The abbreviations used are: ERα, estrogen receptor α; 5-aza-dC, 5-aza-2′-deoxycytidine; MBD, methyl-CpG-binding domain; Dnmt, DNA methyltransferase; HDAC, histone deacetylase; MeCP2, methyl-CpG binding protein 2; MSP, methylation-specific PCR; TSA, trichostatin A; RT-PCR, reverse transcription-PCR.

intron of ER and the first intron of *β-actin* genes, yielding 470 and 400 bp of PCR products respectively, were described previously (6). PCR products were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Quantitative Competitive PCR Analysis of ER Reactivation. A quantitative assay was performed to determine the level of ER mRNA in TSA- and vehicle-treated MDA-MB-231 cells as compared with the expression levels in the known ER-positive MCF-7 and T-47D cells using the method of Wang and Rowley (14). This assay involves coamplification of a wild-type target cDNA (wER) of unknown amount and a competitive template (cER) in known amounts. A truncated competitive template was generated with a sense primer containing a 22-bp deletion (primer 2) and the same antisense primer (primer 3) as the wild-type (Fig. 2*A*). After amplification, the competitive template was separated, gel purified, and quantified by Spectrophotometer DU 640 (Beckman, CA). Thus, target wild-type and known amounts of competitive templates can be coamplified with the pair of wild-type primers (primers 1 and 3) and differentiated by size. Because the most accurate results are obtained when wild-type and competitive templates are amplified at nearly equivalent concentrations, resulting in the signal ratio of wER:cER equivalent to 1, we first performed an initial titration in log and then in 2-fold dilutions to determine the approximate concentration of the wild-type ER cDNA in our experimental samples. RNAs under comparison were simultaneously reversibly transcribed to achieve equal efficiency for reverse transcription. The PCR reactions were carried out with 0.5 μM of wild-type sense and antisense primers for 35 cycles. The wild-type and competitive PCR products were fractionated on 2.5% agarose gel, stained with ethidium bromide, and scanned by Densitometer (EagleSight Software of Eagle Eye II Imaging System; Stratagene, La Jolla, CA). The ratio between wild-type and control templates was determined and used to calculate the amount of target wild-type cDNA because the input of competitive template is known.

Genomic DNA Isolation and MSP Analysis of *ER* CpG Island. DNA was isolated by standard phenol-chloroform extraction. Isolated DNA was subjected to modification by sodium bisulfite to convert unmethylated cytosines but not methylated cytosines to uracil as described previously (15). Methylation status of the bisulfite-modified DNA at the *ER* locus was characterized by methylation-specific PCR using a method described previously (5).

DNase I Sensitivity Assay. This assay was performed according to the method of Keshet *et al.* (16) using cells from TSA- or vehicle-treated MDA-MB-231 and MCF 7 cells (1×10^7 cells/each) with the following modifications. The isolated DNA was digested with *Eco*RI, the recognition sites of which flank the ER promoter region to yield a 3.1-kb fragment that was separated by 1% agarose gel electrophoresis. DNAs blotted on nylon membrane were probed with a PCR-amplified, 561-bp DNA fragment corresponding to the *ER* CpG island (Fig. 4*A*). The sense and antisense oligonucleotides used to amplify the fragment are 5′-AGACCAGTACTTAAAGTTGGAGGCC-3′ and 5′-GGGAAACCCCCCAGG-3′. The amplified DNA was cloned into pCR2.1-TOPO vector (Invitrogen, CA) according to the manufacturer's protocol. Colonies containing amplified DNA sequence, determined by Mini-Prep (Promega Wizard Mini-Prep kits), were grown and purified. The purified plasmids were sequenced via automated sequencing (Johns Hopkins Sequencing Core Facility). The specific *ER* CpG probe was prepared from sequence-confirmed plasmid and labeled with bio-16-dUTP (Boehringer Mannheim, IN) by PCR using the above-mentioned primer set. The 3.1-kb DNA band containing the *ER* promoter region was visualized by chemiluminescence using a streptavidin-conjugated, alkaline phosphatase-catalyzed substrate, CDP-star. The band signals that reflect the resistance to DNase I were quantified by densitometry (Stratagene), and the cumulative DNase I sensitivity was calculated as follows. The band density at each DNase I concentration was divided by the density of the control band and then multiplied by 100%. This value was subtracted from 100% to yield the percentage of DNase I sensitivity. The sum of the percentage of DNase I sensitivity at each dose was considered to be the cumulative DNase I sensitivity.

Results and Discussion

A growing body of data demonstrates the importance of histone acetylation and deacetylation and corresponding structural alteration of chromatin in gene transcriptional regulation (7, 8, 10). The ER-negative cell line, MDA-MB-231, the *ER* CpG island of which is densely methylated, was used as a cell model to test whether HDAC activity contributes to repression of ER expression in ER-negative breast cancer cells. TSA, a specific and potent HDAC inhibitor, was used as a pharmacological tool. Exposure of MDA-MB-231 cells to increasing concentrations of TSA led to induction of ER mRNA synthesis in a dose-dependent manner (Fig. 1*A*). A detectable level of ER mRNA, as demonstrated by a RT-PCR product with predicted 470-bp size, was noted after treatment with 50 ng/ml (160 nM) TSA for 48 h, and ER transcript was clearly present after 100 ng/ml (330 nM) TSA for the same duration. A time course analysis showed that a weak signal could be seen after 24 h of 100 ng/ml TSA treatment, whereas ER transcript was readily observed after 48 or 72 h (Fig. 2*B*). Multiple experiments were done to achieve optimal conditions for TSA induction of ER mRNA in MDA-MB-231 cells. These showed that maximal ER reactivation was achieved with 100 ng/ml TSA for 48 h using an initial seeding density of 8×10^3 cells/cm^2. Higher inoculating cell densities reduced ER transcript signal (data not shown).

To ascertain whether HDAC activity could play a role in repression of ER expression more generally, the dose response and time course studies described above were extended to other ER-negative human breast cancer cells lines with methylated ER CpG islands. As shown in Fig. 1*C*, TSA treatment led to re-expression of ER mRNA in all three ER-negative cell lines tested. Optimal ER gene re-expression was observed after treatment of MDA-MB-231 cells with 100 ng/ml (330 nM), Hs578t cells with 400 ng/ml (1.32 μM), and MCF-7/Adr^R with 25 ng/ml (82.5 nM) TSA for 48 h. Therefore, TSA treatment consistently induced ER re-expression in the panel of ER-negative cell lines, supporting a role for HDAC in *ER* gene silencing.

A quantitative competitive PCR assay was used to assess the magnitude of TSA-induced ER mRNA transcript in MDA-MB-231 cells. Fig. 2*A* shows the design of the primer sets used, and validation of the quantitative competitive PCR is shown in Fig. 2*B*. As shown in Fig. 2*C*, a 5-fold increase in ER transcript was obtained after TSA exposure in MDA-MB-231 cells (100 ng/ml for 48 h). This effect was specific for the methylated ER promoter because TSA treatment (50 or 100 ng/ml for 48 h) of ER-positive, unmethylated MCF-7 cells had

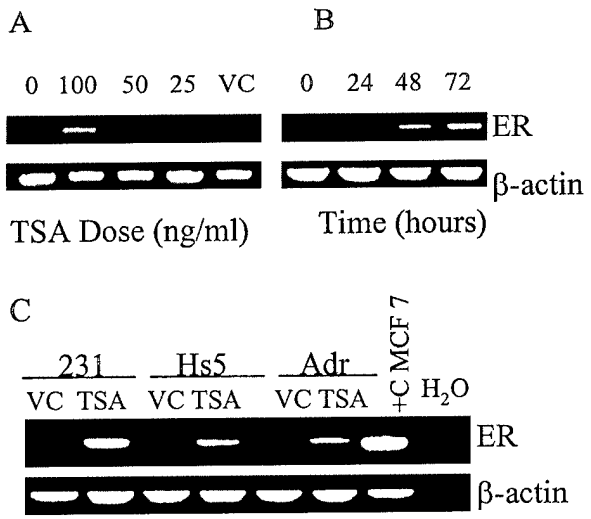

Fig. 1. *Upper panels: A,* dose response of TSA on ER transcription in MDA-MB-231 cells analyzed by RT-PCR. *B,* time course of TSA (100 ng/ml) on re-expression of ER mRNA for 48 h in MDA-MB-231 cells. *C,* RT-PCR analysis of ER mRNA re-expression by TSA for 48 h in a panel of known ER-negative breast cancer cell lines. The ER-positive prototype, MCF-7, was used as a RT-PCR-positive control. *Lower panels:* β-actin RT-PCR product provides a control for the amount of intact RNA used in the reactions. *VC,* vehicle-treated; *231,* MDA-MB-231; *Hs5,* Hs578t; *Adr,* MCF-7/Adr.

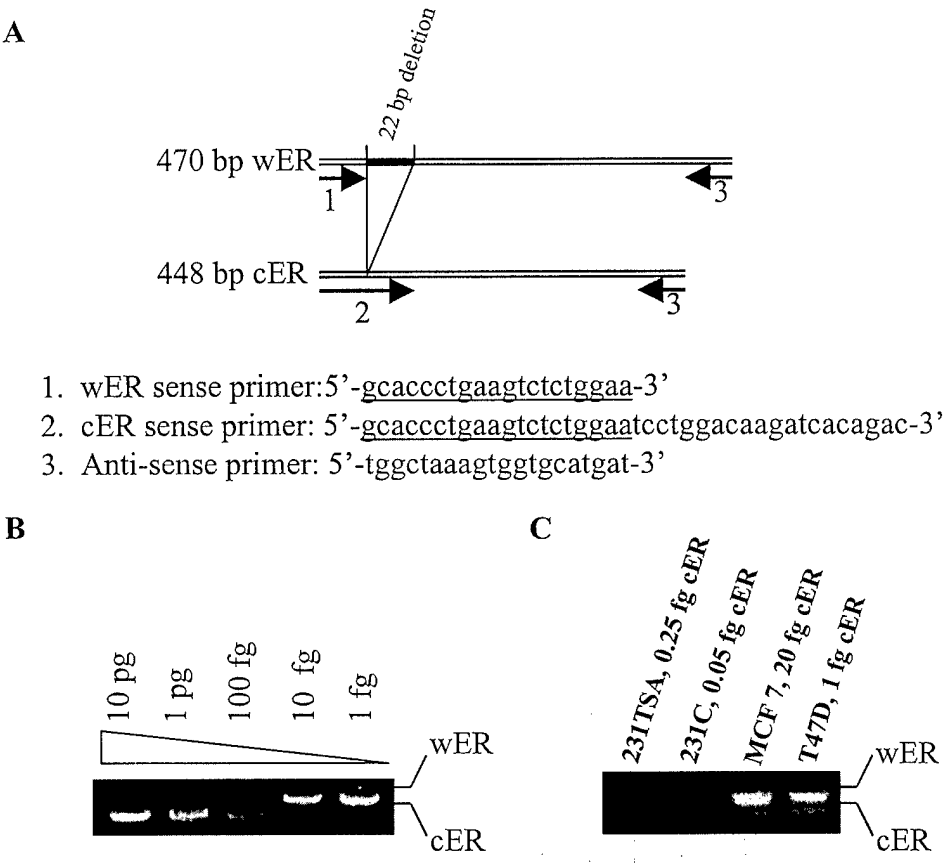

Fig. 2. Quantitative competitive PCR analysis of ER expression in breast cancer cells. *A*, primer design. As shown above, the competitive sense primer 2 is a 40-bp oligonucleotide that harbors a 22-bp deletion in the middle of the primer. Its sequence at the 5′ end is the same as the wild-type sense primer *1*. Thus, primers 2 and *3* amplify cER, but wild-type primer pairs *1* and *3* amplify both wER and cER. *Arrow bars*, locations of sense and antisense primers. *wER*, wild-type ER; *cER*, competitive ER. *B*, validation of the competitive PCR. wER and cER from MCF-7 cells were amplified with the wild-type primers using different dilutions of competitive template DNA. The cER PCR product decreases and the wER PCR product increases when decreasing known amounts of cER and a constant but unknown amount of wER cDNA is used for PCR (see "Materials and Methods"). The amount of wER can be ascertained from the titration curve at the point where an equal amount of cER and wER is, in this example, between 100 and 10 fg. *C*, using this quantitative assay, TSA treatment (100 ng/ml for 48 h) of MDA-MB-231 cells induced a 5-fold increase in ER transcript. Equivalence of wER/cER signal was seen at 0.25 fg cER for TSA-treated cells and 0.05 fg cER for control cells (signal ratio of wER:cER = 1 in both TSA-treated and control cells; see "Materials and Methods"), thus demonstrating a 5-fold induction. Using the same calculations (signal ratio of wER:cER multiplied by amount of cER), the TSA-induced transcript reached about 1 and 10% of that found in ER-positive MCF-7 and T-47D cells, respectively. A representative example of four experiments that gave similar results is shown.

no effect on the level of ER mRNA expression using the same quantitative assay (data not shown). However, TSA treatment of MDA-MB-231 cells did not restore ER mRNA expression to the levels seen in cell lines with endogenous ER expression, as shown in Fig. 2*C*. Quantitative assay suggested that the level of ER transcript seen with TSA treatment of MDA-MB-231 cells represented about 1 and 10% of that seen in the ER-positive MCF-7 and T-47D cell lines, respectively. Several possibilities might account for this:

(*a*) It is possible that only partial reactivation is seen because only a fraction of cells responded to the treatment. Indeed, a similar pattern of partial reactivation was seen in MDA-MB-231 cells exposed to a demethylating agent, 5-aza-dC, in our previous study (6).

(*b*) It has been shown that a component of the repression mediated by MeCP2 transcriptional repression domain is partially HDAC independent; mSin3A could retain some ability to repress transcription, even in the absence of associated HDACs (7).

(*c*) Simultaneous inhibition of several components in the methylation-associated repressive complexes might be necessary to achieve maximal reactivation of the repressed genes (12).

The ability of TSA to reactivate ER expression raised the question of whether the *ER* CpG island remained methylated. Our previous studies with 5-aza-dC demonstrated that ER re-expression was associated with demethylation of the *ER* CpG island. However, a parallel study of the progesterone receptor in progesterone receptor-negative human breast cancer cells showed that the ligand-bound ER could overcome methylation-related repression of the progesterone receptor, even in the continuing presence of a methylated progesterone receptor CpG island (17). We therefore examined the methylation status of the *ER* CpG island in TSA-treated MDA-MB-231 cells using a sensitive MSP assay that allowed examination of methylation status across the *ER* CpG island (Fig. 3*A*). As shown in Fig. 3*B*, the *ER* CpG island was completely methylated across the entire CpG island in MDA-MB-231 cells treated with vehicle or TSA (100 ng/ml for 48 h). A single primer set, ER 5, was used to confirm this finding in the other TSA-treated, ER-negative human breast cancer cells, Hs578t and MCF-7/Adr^R (Fig. 3*C*). As expected, the unmethylated ER-positive, MCF-7 cells demonstrated an unmethylated pattern using all four primer sets that span the *ER* CpG island. In summary, TSA treatment of ER-negative breast cancer cells can lead to re-expression of ER mRNA without an apparent alteration in the methylation status of the *ER* CpG island.

Because acetylated histones are generally associated with transcriptionally active chromatin whereas deacetylated histones are often found in conjunction with an inactive chromatin state (18), we next studied whether HDAC inhibition could alter chromatin structure at the *ER* gene locus. Because nuclease susceptibility is one of the characteristics of active chromatin (19), we used a DNase I sensitivity assay to examine chromatin conformation of the *ER* gene in ER-negative MDA-MB-231 cells in the presence or absence of the HDAC inhibitor, TSA. Cells were treated with 100 ng/ml of TSA for 48 h, a treatment course shown previously to result in optimal re-expression of ER mRNA. Equal amounts of purified nuclei from control and TSA-treated MDA-MB-231 cells were exposed to increasing concentrations of DNase I as described in "Materials and Methods." Nuclei isolated from ER-positive MCF-7 cells served as a DNase I accessible control. As expected, the *ER* locus in MCF-7 cells was a highly DNase I sensitive region, whereas the *ER* locus in control MDA-MB-231 cells was relatively resistant to DNase I digestion (Fig. 4*B*). TSA treatment of MDA-MB-231 cells resulted in an ~2-fold increase in DNase I sensitivity (Fig. 4*C*), suggesting that inhibition of HDAC activity leads to a more open chromatin conformation, even in the presence of CpG island methylation.

Recently, the interaction between DNA methylation and histone deacetylation linked by methyl-binding proteins (MBDs), or the direct interaction of Dnmt1 with HDAC as well as other corepressors, has

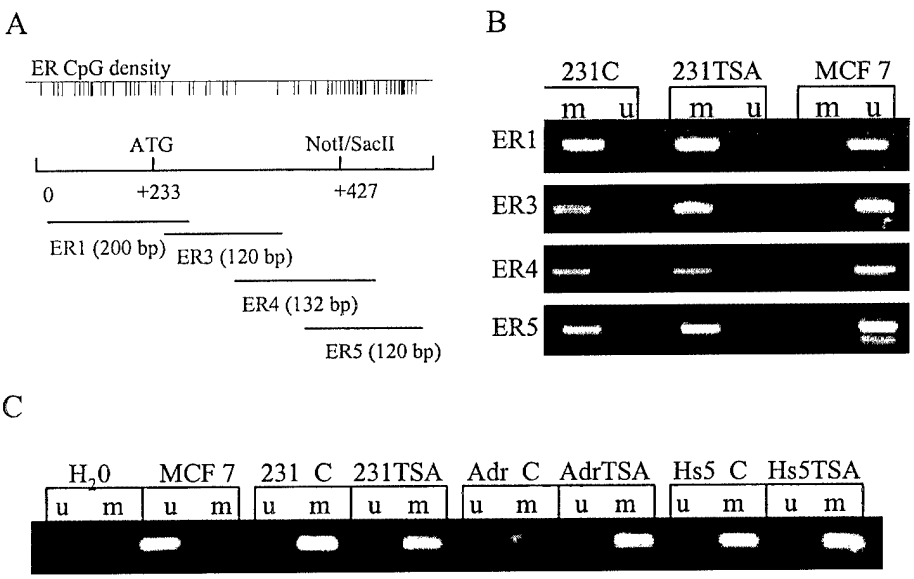

Fig. 3. MSP analysis for the *ER* CpG island in TSA-treated ER-negative breast cancer cells. *A*, map of *ER* CpG island and location of ER MSP primers. *B*, MSP analysis of *ER* gene CpG island in TSA-treated MDA-MB-231 cells. Both control (231) and TSA-treated (231 TSA) MDA-MB-231 cells showed evidence of *ER* CpG methylation across primer sets examined. DNA from the ER-positive MCF-7 cell line was used as an unmethylated control. *C*, MSP analysis of *ER* gene CpG island by using primer set ER 5 in a panel of ER-negative breast cancer cells. Both vehicle- and TSA-treated cell pairs (MDA-MB-231 C and TSA; Hs578t C and TSA; and MCF-7/Adr^R C and TSA) showed methylated pattern, whereas ER-positive MCF-7 showed unmethylated pattern (MCF 7). Distilled water instead of bisulfite-treated genomic DNA was used as MSP-negative control (H_2O). *m*, methylated products; *u*, unmethylated products.

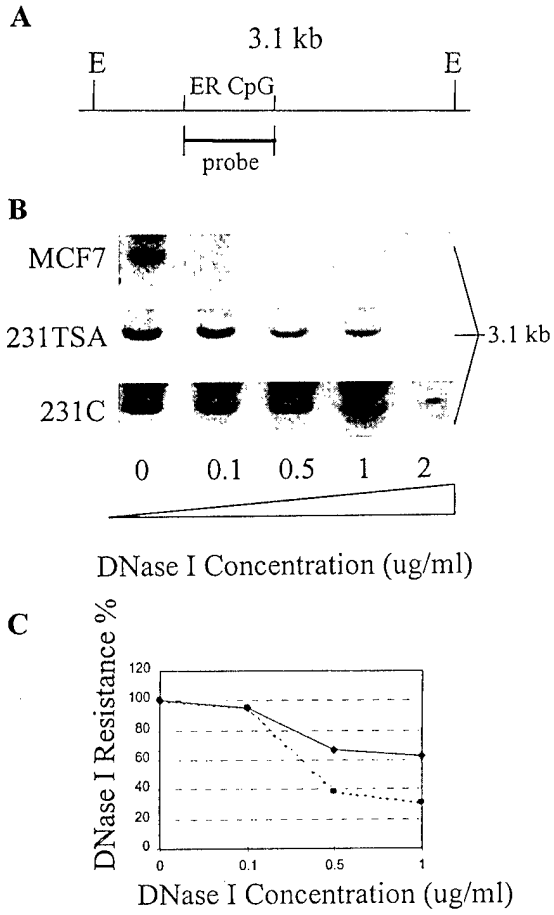

Fig. 4. Chromatin conformation at *ER* gene locus in breast cancer cells analyzed by DNase I sensitivity assay. *A*, map of the *ER* promoter region flanked by *Eco*RI (*E*), showing the location of the *ER* CpG island in the region and probe position. *B*, DNase I digestion profiles with the indicated concentrations of DNase I in TSA-treated (100 ng/ml for 48 h (*231TSA*) and vehicle-treated (*231C*) MDA-MB-231 cells are shown. A highly DNase I sensitive region was found in MCF-7 cells (*MCF7*). Presented is one of three experiments that showed similar results. *C*, quantitative analysis of DNase I sensitivity at 48 h after TSA treatment in MDA-MB-231 cells. The concentration of DNase I was plotted against the percentage of DNase I resistance. Densitometric analysis demonstrated a 1.8-fold increased sensitivity in TSA-treated (●, 231 TSA, cumulative DNase I sensitivity of 135) *versus* vehicle-treated (◆, 231C, cumulative DNase I sensitivity of 75) MDA-MB-231 cells (see "Materials and Methods").

been an area of active study. More recently, a nucleosome-stimulated ATPase Mi2, a part of chromatin remodeling machinery, was also shown to bind the methylated DNA through MBD3 and deacetylase in *Xenopus laevis* and mammalian cells, further illustrating the role of HDAC on gene transcription regulation (8). In addition to evaluating the role of HDAC in *in vitro* studies, it is of importance to study its role in silencing endogenous methylated genes. In some cases, HDAC inhibition alone seems to be sufficient to reactivate a methylated gene. For example, both sodium butyrate and TSA can restore transcription from methylated and silenced plant rRNA genes (20). Also, reactivation of transcription of the methylated *FMR*1 gene was achieved by treatment with 4-phenylbutyrate, sodium butyrate or TSA (21). However, in other cases, both demethylation and HDAC inhibition appear to be necessary. For example, certain hypermethylated genes like *MLH1, TIMP3, CDKN2B,* and *CDKN2A* can be transcriptionally activated in colon cancer cells by TSA only after Dnmt1 inhibition by 5-aza-dC, suggesting an important role of Dnmt1 in transcription repression although recruiting HDAC is essential (12).

ER is a critical growth-regulatory gene in breast cancer, and its expression status is tightly linked to the prognosis and treatment outcome of breast cancer patients. Thus, it is important to understand its regulation. Our work suggests that histone deacetylation and DNA methylation may both play a role in ER transcription, and further studies will focus on the effects of TSA on ER protein expression. This is critical because it is possible that activation of the silenced ER by HDAC inhibition could open a new avenue for management of a subset of advanced breast cancer with hormonal resistance. Studies using primary breast cancers have shown that the antiestrogen, tamoxifen, confers a benefit to women whose breast cancer expresses ER by immunohistochemistry in as few as 1–10% of tumor cells. Thus, even partial re-expression of ER could be of clinical benefit (22).

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Role of DNA Methylation and Histone Acetylation in Steroid Receptor Expression in Breast Cancer[1,4]

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DNA methylation is an epigenetic modification that is associated with transcriptional silencing of gene expression in mammalian cells. Hypermethylation of the promoter CpG islands contributes to the loss of gene function of several tumor related genes, including estrogen receptor α (ER) and progesterone receptor (PR). Gene expression patterns are also heavily influenced by changes in chromatin structure during transcription. Indeed both the predominant mammalian DNA methyltransferase (DNMT1), and the histone deacetylases (HDACs) play crucial roles in maintaining transcriptionally repressive chromatin by forming suppressive complexes at replication foci. These new findings suggest that epigenetic changes might play a crucial role in gene inactivation in breast cancer. Further, inhibition of DNA methylation and histone deacetylation might be a therapeutic strategy in breast cancer, especially for those cancers with ER and PR negative phenotypes.

KEY WORDS: Breast cancer; DNA methylation; histone acetylation; steroid receptor.

INTRODUCTION

DNA Methylation and Cancer-Related Gene Expression

Approximately 3–5% of the cytosine residues in the human genome are methylated (1). Seventy to eighty percent of these 5-methylcytosines are located in clusters of CpG dinucleotides, termed CpG islands, typically found in the 5' promoter region and first exon of certain genes (2). This methylation modification is essential not only in mammalian development, but also in epigenetic regulation of gene expression, including genomic imprinting and X chromosome inactivation (3–5). Methylation of a CpG island is frequently associated with loss of expression of the target gene. Recent studies have provoked increasing interest in the role of DNA hypermethylation in tumorigenesis through its ability to alter the expression of tumor suppressor genes (6).

The DNA methylation reaction is catalyzed by a family of DNA methyltransferases (DNMTs) by use of the universal methyl donor, S-adenosylmethionine. Three distinct DNMTs have been identified in mammalian cells so far, namely DNMT1, DNMT2 and DNMT3. DNMT1 is constitutively expressed in mammalian cells and its function is to maintain the identical methylation pattern after DNA replication (7). That this function is critical is suggested by the finding that Dnmt1 gene knockout mice demonstrated global DNA demethylation and embryonic lethality (8, 9). On the other hand, somatic knockout of the DNMT1 gene in human colorectal carcinoma cells led to markedly decreased cellular DNMT activity, but only

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[4] Abbreviations: estrogen receptor α (ER); progesterone receptor (PR); DNA methyltransferase (DNMT); histone deacetylase (HDAC); histone acetyltransferase (HAT); methylation-specific polymerase chain reaction (MSP); 5-azacytidine (5aza); 5-aza-2'-deoxycytidine (deoxyC); trichostatin (TSA); retinoic acid (RA); acute promyelocytic leukemia (APL).

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a 20% decrease in overall genomic methylation was observed, mainly at juxtacentromeric satellites. Most of the gene-specific CpG islands analyzed remained fully methylated and silenced (10). These results suggest that DNMT1 might not be the only critical enzyme in maintaining human genome methylation status, and its exact role in mammalian development and gene transcription regulation needs to be further elucidated. DNMT2 is expressed at low levels in most adult tissues examined but recombinant mouse DNMT2 has no DNA methyltransferase activity (11–13). Thus the importance of DNMT2 is not clear. DNMT3 does have *de novo* methyltransferase activity and is highly expressed in embryonic stem cells. It has two isoforms, namely DNMT3A and DNMT3B. Unlike DNMT2, recombinant mouse DNMT3A and 3B can methylate cytosine residues in various native and synthetic DNA templates (14). DNMT3A expression is ubiquitous. It can be readily detected in most adult tissues, whereas DNMT3B expression is highly elevated in several tumor cell lines, including leukemia, melanoma, and colorectal cancer cell lines, to a level comparable to DNMT1 in these cell lines (15). How other DNMT family members, such as DNMT2, DNMT3A, DNMT3B, or other novel methylating proteins contribute to CpG methylation during normal development or tumorigenesis needs to be addressed in future study.

Methylation patterns in tumors are perplexing. In general, the level of 5-methylcytosine in tumor cells is lower than that in normal cells (16, 17). However, this global hypomethylation is observed in conjunction with regional hypermethylation at CpG islands, in turn associated with transcriptional inactivation of an increasing number of cancer-related genes. Thus far, a variety of genes, including tumor suppressor genes, DNA mismatch repair genes, cell cycle related genes, hormone receptors and tissue or cell adhesion molecules have been reported to be regulated by promoter CpG methylation. The inactivation of gene expression by aberrant CpG island hypermethylation is supported by studies using the demethylating agents, 5-azacytidine (5aza) and 5-aza-2′-deoxycytidine (deoxyC). Application of these agents to several cancer cell lines has been shown to demethylate CpG islands and reactivate expression of the previously silenced genes. In addition, recent studies suggest that hypermethylation of a CpG island not only can silence the gene it regulates, but also facilitate genetic alterations in tumor progression. $P16^{INK4a}$ (18–21), MLH1 (22, 23), and GSTP1 (24, 25) are the best examples to support this "facilitation hypothesis." Accumulating evidence suggests that hypermethylation of these three genes occurs in the early stages of tumorigenesis, predisposing cells to later genetic instability, which then contributes to tumor progression. Thus it appears that both epigenetic and genetic changes can contribute to the carcinogenic process.

Histone Acetylation, Chromatin Stability and Gene Expression

Chromatin structure and gene transcription are regulated partially by histone acetylation. During the S phase of the cell cycle, histone acetyltransferases (HAT) transfer an acetyl moiety to the ε-amino group of the amino acid, lysine, on histones, leading to neutralization of the positive charge and reduced affinity of histone for DNA. The ultimate consequence of this acetylation modification is the transformation of a tight-coiled inactive chromatin structure into a loose, transcriptionally active one (26). This process is reversed by histone deacetylation mediated by histone deacetylases (HDAC) during the G2 phase of the cell cycle. Deacetylated histones expose their positive charges to negatively charged DNA, leading to a condensed inactive chromatin structure. HDAC seems to play a role in gene silencing as well as in transcriptional activation (27, 28). In addition, HDACs are also involved in cell differentiation, cell-cycle arrest, apoptosis, chemosensitization, radiosensitization, antitumor effects and up-regulation of MHC class I (29). Three families of HDAC have been characterized so far, *S. cerevisiae RPD3p* (mammalian equivalents HDACs 1, 2 and 3), *S. cerevisiae HDA1p* (mammalian equivalents HDACs 4, 5 and 6), and *Zea mays HD2* (29). The understanding of these deacetylases, especially the roles of HDAC1 and 2 in transcription regulation, is expanding.

Like DNA methylation, histone acetylation has recently been associated with tumorigenesis. For example, HAT CBP (a CREB-binding protein) is fused to the MLL gene in cases of acute leukemia or myelodysplasia secondary to therapy with drugs targeting DNA topoisomerase II (30, 31). This novel fusion protein leads to dysregulated histone acetylation that might contribute to the leukemogenic process (31). Histone acetylation may also play a role in breast cancer. The carboxy-terminal domains of proteins encoded by the breast cancer susceptibility genes, BRCA1 and BRCA2, can interact with Rb and Rb binding proteins as well as HDAC1 and HDAC2 (32). It has been proposed that the recruitment of HDACs

by BRCA proteins could cause gene deregulation in the progression of hereditary breast cancer (33, 34). For additional discussion of BRCA1 and BRCA2, see in this issue the article by Mielnicki *et al.* (35).

Molecular Mechanisms for the Epigenetic Regulation of Gene Expression

It appears that both DNA methylation and histone acetylation are involved in epigenetic regulation of gene expression in normal mammalian development as well as in tumorigenesis through their ability to modify chromatin structure during transcription. It also has been demonstrated that methylated inactive genes are associated with underacetylated histones whereas unmethylated active genes are linked to hyperacetylated histones (36, 37). These observations raise the following questions. How do DNA methylation and histone acetylation inhibit transcription? Do these processes interact with each other or act separately during this process? Are there any other factors involved?

Recent studies suggested that DNMT1 can form a repressive transcription complex at replication foci with HDAC2 and a newly identified protein, DMAP1 (DNMT1 associated protein), at the noncatalytic amino terminus of DNMT1. DMAP1 has intrinsic transcription repressive activity and interacts with DNMT1 throughout S phase, whereas HDAC2 joins the complex only during late S phase. These findings indicate that there is a connection between DNMT1 and HDAC, and histone deacetylation mediated by HDAC occurs after DNA replication (38). In another study, DNMT1 has been shown to form a transcription repressive complex with Rb, E2F1 and HDAC1 (39). Therefore, DNMT1, in addition to its function of maintaining CpG methylation, has direct inhibitory effects on transcription by formation of a repressive complex during DNA replication.

DNA METHYLATION AND HISTONE ACETYLATION IN REGULATION OF ER AND PR EXPRESSION IN BREAST CANCER

ER and PR Expression in Normal Mammary Gland and Breast Cancer

Estrogen and progesterone and their receptors play important roles in the development and function of the mammary gland as well as other female reproductive organs. At puberty, the hypothalamus and pituitary gland stimulate the ovary to secrete estrogen that is responsible for the proliferation of the mammary epithelial cells and elongation and branching of mammary ducts. Progesterone is synthesized by the corpus luteum and placenta to promote the growth of mammary lobuloalveolar structures, especially during pregnancy in preparation for milk secretion after parturition (40). Although estrogen and progesterone receptors are expressed at very low basal levels in normal human mammary epithelial cells after a woman reaches sexual maturity (41, 42), they seem to play an important role in the initiation, progression and prognosis of breast cancer. They are also good predictors for endocrine therapy (43). Approximately two-thirds of breast cancers express ER transcript and synthesize ER protein at levels higher than in normal breast tissues and half of these ER-positive tumors express both ER and PR protein (ER+/PR+) (43). These tumors tend to be more differentiated and are more responsive to hormonal therapies. One-quarter of all breast cancers lack both ER and PR (ER−/PR−). In general, these tumors are associated with poorer differentiation, higher growth fraction, and worse clinical outcome than ER+/PR+ breast cancer (43). These cancers are estrogen-independent and rarely respond to hormonal therapies. Of note, up to one-third of initially ER+ tumors can evolve to an ER− status during tumor progression and become resistant to hormone therapy (44).

ER Methylation and DNMT Expression in Breast Cancer

The human ER cDNA and gene were cloned in 1986 (45–47). This 140 kb ER gene has eight exons and is located on chromosome 6q25.1. Since then, efforts have been devoted to the possible molecular genetic mechanisms for the loss of ER expression in breast cancers. Genetic changes, such as insertions, deletions, rearrangements, or point mutations of the ER gene were extensively sought in breast cancer cells since these in-frame or out-of-frame sequence alterations would lead to either absence of ER expression or expression of an unstable mutant that might be degraded shortly after protein translation by the ubiquitin protease system. Although several sequence mutations for the ER gene were identified and shown to be related to decreased ER expression and estradiol binding, they are uncommon and cannot explain the loss of ER expression in a significant fraction of human breast cancers (40). This finding suggests that mechanisms other than genetic changes

Table I. Frequency of ER Gene Methylation of Primary Breast Human Tissues by Use of Southern Blot Analysis[a]

Genotype	ER gene methylation at the *notI* site
ER+/ PR+ tumor	0/29 (0%)
ER+/ PR− tumor	0/24 (0%)
ER−/ PR− tumor	9/39 (25%)
ER−/ PR− metastases	2/2 (100%)
Normal breast	0/9 (0%)

[a]Adapted from Ref. (51).

might also contribute to the loss of ER expression in breast cancer.

One extensively studied epigenetic mechanism that might lead to loss of ER expression is CpG methylation. Interestingly, the ER gene has a CpG island in its promoter and first exon regions marked by a clustering of sites for methylation-sensitive restriction endonucleases (46, 48) (Fig. 1). In addition, absence of ER protein expression in human breast cancer specimens is frequently associated with loss of ER transcript (49). Those findings raise the possibility that absence of ER expression is associated with ER gene hypermethylation.

We have addressed this hypothesis using human breast cancer cell lines as a model system. Ottaviano *et al.* (50) reported that ER− human breast cancer cells displayed extensive methylation of the CpG island in the 5' promoter region of the estrogen receptor gene, in association with silencing of ER gene expression. The 6.3 kb ER mRNA transcript was detected easily in three ER+ cell lines (MCF-7, T47D and ZR75-1), but none was detected in three ER− cell lines (Hs578t, MDA-MB-231 and MDA-MB-468). Southern blot analysis using methylation-sensitive restriction enzymes showed that the *ER* CpG island was methylated at a critical *NotI* site in multiple ER-negative breast cancer cell lines. As expected for an autosomal gene, the ER gene was unmethylated at the *NotI* site in the CpG island in all normal tissues studied including breast epithelium. In addition, the ER gene was unmethylated at multiple other restriction sites in its CpG island in all ER+ cell lines studied, but these sites were frequently methylated in ER-negative cell lines. Of note, the major DNA methyltransferase, DNMT1, was highly expressed in ER− cell lines at the mRNA level as well as the protein and enzymatic activity levels. Careful assessment of the relationship between ER expression and DNMT1 showed that DNMT1 protein expression was correlated with S-phase fraction in ER-positive cell lines, but not in ER-negative cell lines. Thus DNMT1 expression was elevated in ER-negative cell lines and was unlinked from cell cycle regulation (51).

A key question is whether these tissue culture findings have any relevance to human breast cancer. Using Southern blot analysis, Lapidus *et al.* (52) reported that the *ER* CpG island was methylated at the *NotI* restriction site in 9 of 39 (25%) of

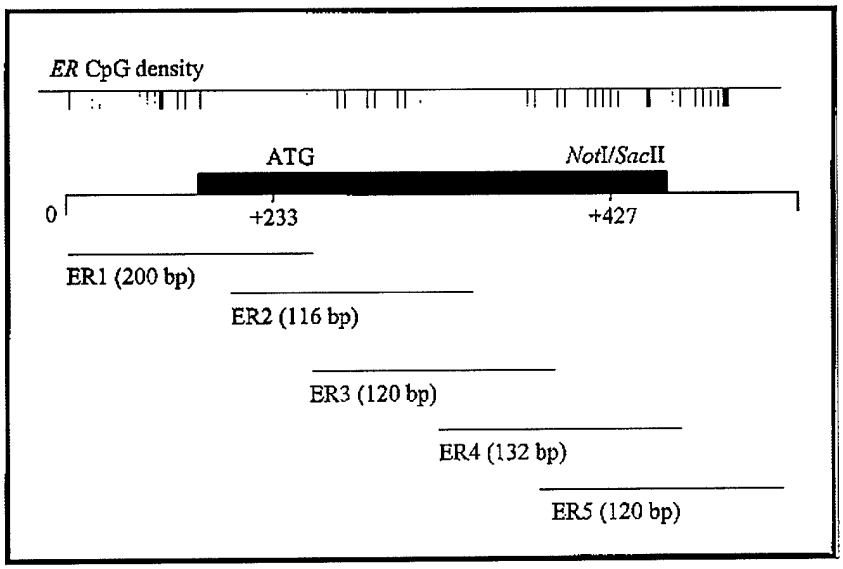

Fig. 1. Map of ER CpG island and locations of PCR products generated by use of specific ER MSP primers. Box represents exon 1 of ER gene.

primary ER– breast cancers but remained unmethylated in 53 ER+ breast cancers and 9 normal breast specimens (Table I). The possible explanations for the different frequency of *ER* methylation in cell lines and tissues include the presence of normal cells in primary cancers, heterogeneity of ER expression within breast cancers, methylation of other sites within the CpG island which were not examined, and the relative insensitivity of Southern blot analysis. These possibilities have been partially addressed through the development of a PCR-based assay termed methylation-specific PCR or MSP, a technique designed to analyze methylation of CpG dinucleotides across the entire *ER* CpG island (Fig. 1). By use of MSP, normal breast tissues and ER+ cell lines shown only an unmethylated product across all 5 primer sets used, whereas ER– cell lines shown methylated products across much of the island (52) (Table II). To confirm that *ER* CpG methylation could also be detected by MSP in primary human breast cancers, a panel of 33 primary breast cancers of known receptor status was studied. All 33 tumors showed at least some degree of methylation at one or more primer sites. By use of a semiquantitative scoring system for elevated CpG density, the percentage of tumors displaying substantial methylation can be calculated for each tumor set defined by receptor status. As shown in Table III, about one-third of ER+/PR+ tumors showed methylation, whereas the percentage increased to 100% in ER–/PR– tumors (53). Taken together, data derived from both our study and others (54) support a correlation between absence of ER expression and aberrant CpG island methylation of ER gene. Whether methylation status actually acts solely or partially to silence ER transcription is a key question.

Table II. ER Gene Methylation of Human Breast Cancer Lines by Use of MSP[a]

Source	ER1	ER2	ER3	ER4	ER5
Normal breast epithelial cells	–	–	–	–	–
ER+ cell lines					
MCF-7	–	–	–	–	–
T47D	–	–	–	–	–
ZR-75-1	–	–	–	–	–
ER– cell lines					
MDA-MB-231	+	+	+	+	+
Hs578t	+	+	±	+	+
MDA-MB-435	±	–	±	+	±
MDA-MB-468	+	±	±	±	±
MCF-7/Adr	+	+	±	±	±

[a]The results of normal breast epithelial cells are representative of five normal breast epithelial samples. "+": methylated; "–": unmethylated; "±": heterogeneous, both methylated and unmethylated PCR products. Adapted from Ref. (53).

Table III. ER Gene Methylation of Primary Human Primary Breast Cancers by Use of MSP

Receptor status	No. of tumors	No. of methylated tumors (percentage)
ER+/PR+	11	4 (36%)
ER+/PR–	11	8 (72%)
ER–/PR–	11	11 (100%)

[a]Adapted from Ref. (53).

Demethylation of the ER Gene Results in Re-Expression of ER

If silencing of ER expression is a function of CpG island methylation of ER gene, it is possible that demethylation should result in the re-expression of ER in ER– breast cancer cells. In accord with this prediction, treatment of the ER– human breast cancer cell line, MDA-MB-231, with the demethylating agents, 5-aza and deoxyC, led to re-expression of ER (55). After treatment with either drug, the DNA from these cells became partially demethylated at several methylation-sensitive restriction enzyme sites, including *Hha*I, *Not*I, and *Sac*II, within the *ER* promoter CpG island. Demethylation correlated with re-expression of the ER gene as detected by reverse transcriptase-PCR (RT-PCR) as demonstrated in Fig. 2 and Western Blot analysis. Functional activity of this ER protein was examined in two ways. First, its ability to activate expression of an endogenous estrogen-inducible gene, PR, was assessed. PR mRNA and protein were induced by estrogen treatment of deoxyC treated MDA-MB-231 or Hs578t cells. Second, the ability of the deoxyC-induced ER to transduce expression of a luciferase reporter gene linked to an estrogen response element (ERE) was assessed. MDA-MB-231 cells were stably transfected with an ERE-tk-luciferase construct with the expectation that deoxyC-induced ER expression would increase luciferase expression. As expected, increased luciferase activity (1.5–5.6 fold in 10 independent clones) over the background level of the untreated cells was observed (54). These results provide powerful evidence that DNA methylation of the *ER* CpG island plays a functional role in suppression of ER expression in ER– breast cancer cells.

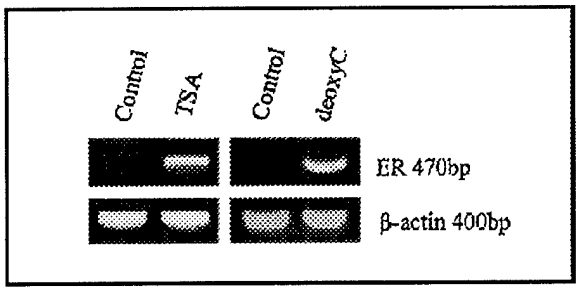

Fig. 2. RT-PCR analysis of ER mRNA re-expression by TSA (100 ng/ml × 2 days) or deoxyC (2.5 uM × 4 days) treatment in ER– MB-MDA-231 cells. β-actin RT-PCR product was used as a control for the amount of RNA used.

DNA Methylation and PR Gene Expression

As discussed in the Introduction, one quarter to one-third of breast cancers lack both ER and PR, whereas another one-third are ER+ but PR–. It also has been demonstrated that the expression of PR requires ER as a transcriptional activator (56). Therefore, the presence of PR in ER+ tumors may be a better predictor of hormone responsiveness than ER alone (43). Interestingly, the PR gene also has a typical CpG island in its first exon. The *PR* CpG island is 1 kb in size, has 70% GC content, and contains a wide array of methylation-sensitive restriction sites. Southern analyses targeted at three methylation-sensitve restriction sites in the *PR* CpG island showed that these sites are not methylated in normal breast tissue specimens but are hypermethylated in 40% of primary tumors that lack PR protein expression (52). Thus the question of why the PR hypermethylation could be responsible for a lack of PR expression was studied in MDA-MB-231 cells which lack both ER and PR (57). Treatment of these cells with the demethylating agent, deoxyC, led to partial demethylation of the *ER* and *PR* CpG islands and was associated with induction of PR protein expression as demonstrated by Western blot analysis. However, Ferguson *et al.* (57), using MDA-MB-231 cells stably transfected with an inducible expression vector for ER, found that the induction of PR gene expression by ligand-bound ER did not require demethylation of the *PR* CpG island. In addition, induction of PR transcription was inhibited by blocking the interaction of ER with SRC-1A, a coactivator of ER function. These results suggested that a transcription factor with the potential to remodel heterochromatin (ER) could activate PR gene expression without altering the methylation status of the CpG island in the PR gene. These results raise the possibility that demethylation and histone acetylation are distinct but complementary mechanisms for destabilizing heterochromatin and activating gene transcription (58).

Histone Acetylation and ER Expression

As discussed earlier, histone acetylation and deacetylation are intimately involved in chromatin structure changes during transcription. In additions, new models of transcriptional suppressive complexes suggest that HDACs and DNMT1 are in direct contact. Recent studies indicate that silencing of a gene by methylation involves the generation of an inactive chromatin structure in which methyl CpG-binding protein (MeCP2) and the adapter protein, mSin3A, recruit histone deacetylase (59, 60). The deacetylation of lysine groups of histones H3 and H4 allows ionic interactions between positively charged lysines and negatively charged DNA, resulting in a more compact nucleosome structure that limits gene activation. The question emerges of whether epigenetic modification of histone acetylation alone could result in re-expression of genes that are inhibited by the transcriptional suppressive complex, or whether both histone acetylation and CpG demethylation are required for this event.

The answers to this question have been variable. For example, HDAC inhibitors like trichostatin (TSA) or phenylbutyrate alone restored retinoic acid receptor α (RARα) expression in retinoic acid (RA) resistant acute promyelocytic leukemia (APL) cell lines as demonstratated by the differentiation of APL cells in the presence of RA (61–63). However, hypermethylated genes, such as MLH1, TIMP3, INK4B (p15) and INK4a (p16), could not be transcriptionally reactivated by TSA alone, but could be re-expressed in colon cancer cells with a combination of TSA and the demethylating agent, 5-aza (58).

To study the role of histone acetylation in ER expression, ER– MDA-MB-231 cells were treated with HDAC inhibitor, TSA. A time- and dose-dependent reactivation of ER mRNA expression was observed (64). As shown in Fig. 2, TSA alone at 100 ng/ml for 2 days could reactivate ER expression as well as deoxyC in ER– MDA-MB-231 breast cancer cell line. By use of quantitative competitive PCR assay, an increase of 5.0 fold of ER transcript expression was reported (64). MSP analysis of the *ER* CpG island showed no

change in its methylation after TSA treatment, suggesting that TSA's effects on ER re-expression were not associated with a change in methylation status. This finding was confirmed via a more rigorous assay, bisulfite genomic sequencing of the *ER* promoter region, a technique which permits the direct examination of the methylation status of each CpG dinucleotide within the promoter region. Multiple clones of control and TSA-treated cells were examined and did not show any change in the methylation status of the ER gene with TSA. A logical extension of these studies will be to examine the effect of combinations of demethylating agents and HDAC inhibitors on expression of ER as well as other methylated genes in breast cancer cells.

DNA Methylation and HDAC Inhibitors as Therapeutic Strategies in Breast Cancer

Abundant evidence indicates that epigenetic mechanisms play pivotal roles in the pathogenesis of cancer. Both DNA methylation and histone acetylation have been shown to be involved in the regulation of expression of cancer-related genes. The studies in breast cancer summarized earlier demonstrated that both demethylating agents and HDAC inhibitors could re-activate expression of a number of important growth regulatory genes in several breast cancer cell lines, thus raising the possibility of using these types of agents as therapeutic strategies for breast cancer.

Two demethylating agents, 5aza and deoxyC, have been studied clinically, particularly in patients with hemoglobinopathies. For example, 5aza has been successfully used to treat patients with β-thalassemia. It has been reported that loss of expression of the γ-globin gene is associated with methylation of its promoter (65). Infusion of 5aza into β-thalassemia patients led to an increase of γ-globin expression and amelioration of anemia (66). Similarly, 5aza can increase HbF production and reduce anemia in sickle cell disease (67). In addition, in an ongoing trial, the efficacy of treatment of nasopharyngeal tumors with 5aza is being evaluated. The promoter of one of the Epstein Barr Virus-related proteins is heavily methylated in nasopharygneal carcinoma. It has been proposed that demethylation of this promoter could lead to reactivation of an EBV latency protein that could then become the target for a secondary immune therapy (R. Ambinder, Johns Hopkins Oncology Center, personal communication). Another possible approach is to use antisense oligonucleotides against DNA methyltransferase to inhibit DNA methylation. It has been shown that antisense oligonucleotides have *in vitro* and *in vivo* antitumor activity and a clinical trial of this approach is in progress (68, 69).

Several HDAC inhibitors have the potential to be used as anti-cancer agents (29). The most plausible and extensively studied are butyrate, TSA, and trapoxin (TPX) and their relatives. The IC_{50} of butyrate for HDAC is in the millimolar range, whereas TSA and TPX act at low nanomolar concentrations. One triglyceride analogue of the short-chain fatty acid butyrate, tributyrin, has been reported to induce transcription of p21, arrest of cells in G2/M and apoptosis in MCF-7 human breast cancer cell lines. (70). Two butyrate derivatives, phenylbutyrate (PB) and phenylacetate (PA), have powerful growth inhibitory effects on several cell types including ovarian and prostate cancers (71). A clinical trial with sodium phenylacetate in patients with thalassemia showed an increase of fetal hemoglobin in some patients (72). Other HDAC inhibitors under study include FR901228, oxamflatin, depudecin, suberoylanilide hydroxamic acid (SAHA), m-carboxycinnamic acid bishydroxamide (CBHA) and apicidin.

A potential strategy is to combine demethylating agents and HDAC inhibitors. From a clinical point-of-view, the potential benefits of this combination could include lowering of drug concentration requirements, shorter periods of drug exposure, and lower toxicity, as well as the possibility of synergy. For example, synergistic effects were observed in a combination study with both a demethylating agent and a HDAC inhibitor in colon cancer cells. If cancer cells were treated with 100 nM 5aza (a dose that had little effect on gene expression if used alone) for 24 hours followed by 300 nM TSA for 24 hours, (again a treatment that was ineffective alone), re-expression of several genes was obvious (58). A key question in development of this strategy will be the specificity and selectivity of gene re-expression patterns and how they might be exploited clinically.

Finally, it should be noted that demethylation and histone acetylation are only two mechanisms for epigenetic regulation of target genes like ER. Other transcriptional modulators might also contribute to the epigenetic regulation of gene expression. For example, it was reported that AP2 transcription factor can trans-activate the cloned human ERα promoter in MDA-MB-231 cells (73).

CONCLUSIONS

The evidence for epigenetic regulation of gene expression in tumorigenesis is accumulating. Two components, demethylation of C5-methylcytosines at the CpG island within the promoter region of target genes and histone acetylation of chromatin, have been studied extensively by use of demethylating agents and HDAC inhibitors. *ER* and *PR*, two critical genes in breast cancer development and treatment, have been reported to be densely methylated in ER– but not ER+ human breast cancer cell lines. Treatment of ER– human breast cancer cells with a demethylating agent or a HDAC inhibitor can lead to reactivation of ER expression in these cells. However, demethylation of *PR* CpG island is not required for PR expression. These data in combination with similar results observed with other critical genes in breast cancer and other types of cancer support the potential for compounds that can modulate epigenetic regulation in the treatment of human cancers.

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DNA methylation in breast cancer

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Abstract

Like all cancers, breast cancer is considered to result in part from the accumulation of multiple genetic alterations leading to oncogene overexpression and tumor suppressor loss. More recently, the role of epigenetic change as a distinct and crucial mechanism to silence a variety of methylated tissue-specific and imprinted genes has emerged in many cancer types. This review will briefly discuss basic aspects of DNA methylation, recent advances in DNA methyltransferases, the role of altered chromatin organization and the concept of gene transcriptional regulation built on methylated CpGs. In particular, we discuss epigenetic regulation of certain critical tumor suppressor and growth regulatory genes implicated in breast cancer, and its relevance to breast cancer diagnosis, prognosis, progression and therapy.

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Introduction

Cytosine methylation and CpG islands in mammals

In vertebrate genomes, methylation of DNA occurs on cytosine residues of the CpG dinucleotides in DNA (Bird 1980). This epigenetic alteration in DNA is heritable but does not alter nucleotide sequence, in contrast to genetic changes (Feinberg 2001). Thus, unlike genetic changes, epigenetic modifications are potentially reversible (Baylin *et al.* 2001). About 3–6% of cytosines are methylated in mammals. Approximately 70–80% of CpG sites in the human genome are methylated (Vanyushin *et al.* 1970, Antequera & Bird 1993, Bird 1995). Cytosine residues in newly synthesized DNA are methylated by DNA-cytosine methyltransferase-1 (DNMT1) (Bestor 1988, Bestor & Verdine 1994). This enzyme transfers a methyl group from the methyl donor, S-adenosylmethionine, to nascent DNA using a hemimethylated DNA template in order to maintain DNA methylation patterns during cell division in mammals. CpG dinucleotides are not randomly distributed throughout the genome. Rather they are frequently clustered into CpG islands, regions that are rich in CpG sites. These islands extend about 0.5–3 kb, occur on average every 100 kb in the genome and are often found in the promoter area of genes (Cross & Bird 1995). Indeed, approximately half of all genes in the human (~45 000 genes) contain CpG islands (Antequera & Bird 1993). DNA methylation plays a role in such diverse functions as gene imprinting (Forne *et al.* 1997, Reik & Walter 1998), X-chromosome inactivation (Heard & Avner 1994, Heard *et al.* 1997), normal development (Li *et al.* 1993, Weiss & Cedar 1997), repression of gene transcription (Keshet *et al.* 1985, Cedar 1988, Chan *et al.* 2000), and the suppression of parasitic DNA sequences (Yoder *et al.* 1997). It is believed to exercise its critical role in gene expression via several routes. First, methylation of a CpG island is associated with loss of transcription of the target gene. Secondly, 5-methylcytosine (5meC) residues are susceptible to deamination to thymine, resulting in a transition mutation (Schmutte & Jones 1998). Thirdly, it is possible that inappropriate hypomethylation could be linked to dysregulated gene induction (Muller *et al.* 2001).

DNMTs

To date, three members of the Dnmt gene family have been identified. Direct sequence analysis has revealed that the *Dnmt* gene family is highly conserved among eukaryotes, suggesting a central role of these proteins for development (Bestor 2000).

DNMT1 is the best known and studied member of the DNMT family. It is primarily a maintenance methylase, that is, it reproduces DNA methylation patterns from hemimethylated DNA during cell division (Bestor 1988). However, there is some evidence that DNMT1 may also have *de novo* methylase activity, at least in *in vitro* systems

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(Laayoun & Smith 1995, Pradhan *et al.* 1997). *Dnmt1* gene knockout mice die in mid-gestation with reduced levels of DNA methylation (Li *et al.* 1992), disrupted imprinting and ectopic X-chromosome activation (Li *et al.* 1993), indicating that maintenance of DNA methylation is pivotal for development.

The human *DNMT1* gene is located at human chromosome 19p13.2 (Yen *et al.* 1992) and encodes a 200 kDa protein whose methyltransferase catalytic domain is located at the C-terminus of the protein. The large N-terminal portion of DNMT1 targets to replication foci through proliferating cell nuclear antigen (PCNA) (Chuang *et al.* 1997). Recent studies have identified new functions for this domain. First, its amino acid (653–730) sequence contains a CXXC motif that interacts directly with histone deacetylases (HDACs), which act to remove acetyl tails from histones in the nucleosome to generate a transcriptionally inactive chromatin structure (Fuks *et al.* 2000). Secondly, through its first 120 amino acids, it binds to a transcriptional co-repressor, DMAP1, that represses transcription independent of HDAC activity (Rountree *et al.* 2000). Lastly, amino acids 416–913 of the N-terminus of DNMT1 interact with the retinoblastoma protein, Rb (Robertson *et al.* 2000). Thus, the N-terminal portion of DNMT1 alone or in collaboration with other co-repressors and recruited HDACs significantly suppresses transcription *in vitro*.

A large body of data demonstrates that DNMT1 activity is elevated in neoplastic cells and this increased activity is associated with increased cell proliferation (El-Deiry *et al.* 1991), tumorigenesis (De Marzo *et al.* 1999) and tumor progression (Issa *et al.* 1993). For example, overexpression of DNMT1 can transform NIH-3T3 mouse fibroblast cells (Wu *et al.* 1993), and inhibition of this enzyme by antisense constructs can induce global DNA demethylation and revert the malignant phenotype (Ramchandani *et al.* 1997). In addition, *fos*-mediated transformation of normal fibroblasts is associated with increased DNMT1 expression and total methylation content in the genome (Bakin & Curran 1999). Finally, it is also reported that elevation of DNMT1 is an essential component of transformation induced by SV40 large T antigen via the Rb pathway (Slack *et al.* 1999).

However, increased DNMT1 expression is apparently not an obligatory feature of malignant cells (Eads *et al.* 1999). Somatic knockout of *DNMT1* expression in human colon cancer cells is not a lethal event. Further, total genomic methylated CpG content was reduced by only about 20% and certain gene-specific CpG island methylation patterns were maintained (Rhee *et al.* 2000). These findings, together with the observation that embryonic stem (ES) cells from *DNMT1* knockout mice are still capable of *de novo* methylation, suggest the possible existence of other DNMTs (Li *et al.* 1992, Lei *et al.* 1996).

One such DNMT, Dnmt2, was isolated by several groups (Okano *et al.* 1998). However, its catalytic domain lacks DNMT activity in the human and it is not discussed further.

Two isoforms of the DNMT3 enzyme family, *de novo* DNMTs 3a and 3b (Dnmt3a and 3b) were recently isolated in the mouse (Okano *et al.* 1999). They methylate CpG dinucleotides of unmethylated and hemimethylated DNA *in vitro*. The two genes are expressed at high levels in ES cells and relatively low levels in adult somatic tissues. Human *DNMT3a* has been mapped to chromosome 2p23 whereas *DNMT3b* maps to chromosome 20q11.2 (Robertson *et al.* 1999, Xie *et al.* 1999).

Disruption of both Dnmt3a and Dnmt3b in mice by gene targeting blocks *de novo* methylation in ES cells and early embryos, but has no effect on maintenance of an imprinted methylation pattern (Okano *et al.* 1999). However, methylation capability is retained after inactivation of either Dnmt3a or 3b, indicating some redundancy in the function of these two *de novo* methylases. Dnmt3b appears to be critical for the methylation of a particular compartment of the genome; loss of DNMT3b catalytic activity by gene mutation in the syndrome of immunodeficiency, centromeric instability and facial anomalies causes demethylation of only specific families of repeated sequences and CpG islands on the inactive X-chromosome (Hansen *et al.* 1999). Human DNMT3a is ubiquitously expressed but DNMT3b is expressed at low levels except in testis, thyroid and bone marrow. Overexpression of both *DNMT3b* and *DNMT3a* appears to characterize multiple types of human tumors (Xie *et al.* 1999). Four spliced forms of DNMT3b with altered enzymatic activity were expressed in a tissue-specific manner (Robertson *et al.* 1999). Future study will be needed to elucidate the possible roles of DNMT3 family members in tumorigenesis, *de novo* tissue-specific gene methylation and transcriptional regulation in somatic tissues.

Altered CpG island methylation, chromatin organization and transcriptional regulation

Much experimental evidence has documented the association of CpG island methylation and gene transcriptional inactivity but only recently have the underlying mechanisms of transcriptional silencing by methylation been partially clarified. One possible mechanism of transcriptional repression is the direct interference by methylation with the binding of sequence-specific transcription factors, such as AP-2, E2F and NFκB to DNA (Hermann & Doerfler 1991). A second possibility is that methylated CpG sequences recruit transcriptional co-repressors like mSin3A, DMAP1, TSG101 or Mi2, thereby contributing to transcriptional repression. Finally, chromatin structure is emerging as an important and more generalized mechanism to silence a variety of methylated tissue-specific and imprinted genes by HDAC family members. The deacetylation of lysine groups

of histones H3 and H4 allows ionic interactions between positively charged lysines and negatively charged DNA, resulting in a more compact nucleosome structure that limits gene activity. The discovery of the family of methyl-CpG-binding proteins provides a mechanistic linkage between DNA methylation and histone deacetylation as mediators of gene transcription. To date, six methyl-CpG-binding proteins including MeCP2, MBD1, MBD2a, MBD2b and MBD3 have been identified in vertebrates (Nan *et al.* 1998, Wade *et al.* 1998, Ng *et al.* 1999, 2000, Snape 2000). The common functional features for these proteins are that they bind to methyl-CpGs in DNA and frequently associate with members of the HDAC family, which currently includes eight distinct members (Ng & Bird 2000). That these processes might collaborate to regulate gene expression is demonstrated by a recent study, showing that multiple hypermethylated genes, such as *MLH1*, *TIMP-3*, *CDKN2B* and *CDKN2A*, could be robustly reactivated by a combination of DNMT1 and HDAC inhibition, suggesting that DNMT1 and HDAC are both essential in the silencing process in these colon cancer cells (Cameron *et al.* 1999). The above observation was confirmed by a very recent study that the known DNA methylation machinery protein, DNMT1 itself, is implicated in forming transcriptionally repressive complexes with HDAC as well as other co-repressors (Rountree *et al.* 2000).

DNA methylation patterns in normal tissues and cancer

Cell type and tissue-specific methylation patterns are established during early development, in part through the action of the *de novo* Dnmt3a and 3b (Okano *et al.* 1999). The sperm genome is extensively methylated while the oocyte genome is not. After fertilization, genes are demethylated and then remethylated before implantation. As the embryoblast differentiates, tissue-specific genes are demethylated in a tissue-specific fashion while housekeeping genes remain demethylated from fertilization through organogenesis (Bestor 1998).

CpG islands are generally unmethylated in normal adult tissues with the exception of transcriptionally silent genes on the inactive X-chromosome and imprinted genes like the H19 gene (Tremblay *et al.* 1995). Conversely, most neoplastic tissues demonstrate whole genomic hypomethylation and local promoter hypermethylation in certain critical tumor suppressor and growth regulatory genes (Baylin *et al.* 1998). The mechanism responsible for this type of pattern remains largely unclear. It is believed that the cell cycle checkpoint gene, p21*WAF1/CIP1*, may play a role in methylation regulation (Baylin *et al.* 1998). Since p21 competes with DNMT1 binding to PCNA, loss of p21 function may increase DNMT1 at replication sites (Chuang *et al.* 1997). In addition, mutation of another cell cycle gene, Rb, may play a role as Rb mutation in its A/B pocket domain might disrupt the function of the transcriptionally repressive protein complex that involves Rb, DNMT1 and HDAC. Mistargeting of DNMT1 could then result (Robertson *et al.* 2000). Together, these observations are beginning to shed light on the paradox of global hypomethylation, increased CpG island hypermethylation, and increased DNMT1 activity in tumor cells.

DNA methylation and genetic instability

DNA methylation changes may ultimately lead to the genetic instability characteristic of cancer in several ways. First, 5meCs serve as sites of transition mutations by the hydrolytic deamination of 5meC to thymine. For example, such mutations frequently occur in the well-known p53 tumor suppressor gene (Magewu & Jones 1994). Similar point mutations characterize the mutations found in several other important genes like Rb, and c-H-ras-1 (Ghazi *et al.* 1990).

Secondly, epigenetic inactivation of certain critical genes in cancer by promoter methylation may predispose to genetic instability (Herman & Baylin 2000). For instance, methylation of *MLH1*, a gene involved in mismatch repair, precedes the MIN + phenotype in sporadic colon, gastric and endometrial cancers (Esteller *et al.* 1999*a*). Further, there is a striking correlation between mismatch repair, genetic instability and methylation capacity in colon cancer cell models (Lengauer *et al.* 1997, 1998). In addition, promoter CpG island methylation and resulting inactivation of the detoxifying π-class glutathione S-transferase (GST) can lead to accumulation of oxygen radicals and subsequent DNA damage. The resulting adenine or guanine mutations are implicated in carcinogenesis in prostate, breast and other tissues (Lee *et al.* 1994, Henderson *et al.* 1998, Matsui *et al.* 2000). Further, a p53-inducible gene, 14–3–3σ is methylated and inactivated in many breast cancers. Loss of its expression may also facilitate the accumulation of genetic damages (Ferguson *et al.* 2000).

Apart from regional hypermethylation of some critical tumor suppressor genes, genome-wide hypomethylation is an important feature in cancer. This also could contribute to genetic instability (Schmutte & Fishel 1999).

Methylation of critical tumor suppressor and growth regulatory genes in breast cancer

A large body of evidence has demonstrated that CpG island hypermethylation is implicated in loss of expression of a variety of critical genes in breast cancer. Some important genes inactivated by methylation in breast cancer are summarized in Table 1 and described below. They fall into several broad categories including cell cycle regulating, steroid receptor, tumor susceptibility, carcinogen

Table 1 Frequency of methylation of representative tumor suppression and growth regulatory genes in breast cancer.

Gene	Function	Incidence (%)	Reference
p16^{INK4a}	Cyclin-dependent kinase inhibitor	15	Herman *et al.* (1995)
14–3–3σ	G2 checkpoint	91	Ferguson *et al.* (2000)
ERα	Steroid receptor	50	Ottaviano *et al.* (1994)
PR	Steroid receptor	40	Lapidus *et al.* (1996)
RARβ2	Steroid receptor	25	Sirchia *et al.* (2000)
BRCA1	DNA damage repair	15	Dobrovic & Simpfendorfer (1997)
GSTP1	Carcinogen detoxification	30	Esteller *et al.* (1998)
E-cadherin	Epithelial cell–cell adhesion	50	Graff *et al.* (1995)
TIMP-3	Inhibition of MMPs	25	Bachman *et al.* (1999)

detoxification, cell adhesion and inhibitors of matrix metalloproteinases (MMPs) genes.

Methylation of cell cycle-related genes in breast cancer

p16/p16^{INK4A}/CDKN2A/MTS *methylation and breast cancer*

The *p16* gene is located on chromosome 9p21. It encodes a cyclin-dependent kinase inhibitor, p16^{ink4A}, that regulates the transition from G1- to S-phase via its effect on Rb phosphorylation (Liggett & Sidransky 1998). The transcription of the *p16*^{INK4A} gene can yield two distinct transcripts (α or β) that code for two functionally distinct proteins, p16^{INK4A} and p19^{ARF}. These two transcripts share identical second and third exons but have distinct first exons (Sharpless & DePinho 1999). Loss of p16^{INK4A} resulting from homozygous deletion, methylation of p16 promoter or point mutation is a common feature of many cancers. Methylation of the 5′ promoter and exon 1 regions is observed in both human breast cancer cell lines (Table 2) and 20–30% of primary breast cancers (Herman *et al.* 1995, Woodcock *et al.* 1999). The methylation phenotype is associated with loss of expression at both mRNA and protein levels although it does not correlate with some important clinical parameters in some relatively small cohort studies. For example, a study of 97 patients with breast cancer showed no association between *p16* methylation and overall or disease-free survival (Hui *et al.* 2000).

Finally, the stepwise inactivation of cyclin D-dependent kinase inhibitor p16^{INK4A} in human mammary epithelial cells (HMEC) is associated with progressive methylation of the *p16* promoter CpG island. This allows HMECs to escape from M0 proliferation block, thereby identifying CpG methylation together with *p16* silencing as a possible contributor to breast tumorigenesis (Foster *et al.* 1998).

14–3–3σ *gene inactivation by methylation*

The 14–3–3σ gene (also known as *HME1*), is localized at chromosome 1p35, and is a member of a gene family responsible for instituting the G_2 cell cycle checkpoint in response to DNA damage in human (Chan *et al.* 1999). Normally expression of σ is induced in response to DNA damage, and it causes cells to arrest in G_2. However, σ protein expression was downregulated in a significant fraction of primary bladder, colon and breast tumors (Celis *et al.* 1999). Studies of the molecular mechanisms responsible for the reduced expression have implicated hypermethylation of the CpG-rich exon 1 region of the gene, instead of genetic alterations such as loss of heterozygosity (LOH) and intragenic mutations in breast cancer (Ferguson *et al.* 2000). DNA from HMECs, immortal MCF-10A and HBL100 cells and two breast cancer cell lines, MCF-7 and MDA-MB-231, were unmethylated at the σ locus. In contrast, Hs578t and MDA-MB-435 cells were fully methylated as demonstrated by bisulfite genomic sequencing and methylation specific PCR (MSP) analyses. The use of 5-aza-2′-deoxycytidine (5-aza-dC) to treat the methylated non-expressing lines *in vitro* led to induction of transcription, further supporting the role of CpG island methylation in its repression. In addition, six DNA samples from microdissected normal mammary epithelial cells demonstrated an unmethylated pattern while 32 samples from microdissected breast carcinomas were methylated. Together, these cell line- and tissue-based studies support a role for methylation in the loss of 14–3–3σ expression in breast cancer.

Methylation of steroid receptor genes in breast cancer

The methylation of three members of the steroid hormone superfamily has been extensively studied in breast cancer models. These include estrogen receptor (ER) α, progesterone receptor (PR) and retinoic acid receptor-β (RARβ).

ERα *methylation and hormone resistance*

Steroid hormones, particularly estrogen, have long been linked to mammary carcinogenesis (Fishman *et al.* 1995). The role of estrogen and its catechol metabolite in breast cancer initiation and promotion is a continuing area of controversy (Yager 2000). That 17β-estradiol stimulates the growth of certain breast cancers via functional ER is well recognized, and endocrine therapy is an established and important part of breast cancer management (Ruiz-Cabello *et*

Table 2 Methylation status of critical tumor suppressor and growth regulatory genes in human breast cancer cell lines.

Cell line	Gene							
	p16	*14–3–3σ*	*ER*	*PR*	*RAR-β2*	*BRCA1*	*GSTP1*	*E-cadherin*
HMECs	U/M	U	U	ND	U	U	ND	ND
HBL-100	U	U	ND	ND	ND	U	ND	ND
MCF-7	D	U	U	U	M	U	M	U
T47-D	M	ND	U	U	U	U	M	U
ZR75-1	U/M	ND	U	U	M	U	M	ND
MDA-MB-231	D	U	M	M	M	U	U	ND
MDA-MB-468	U	ND	M	M	M	U	ND	U
MDA-MB-435	ND	M	M	M	U	U	ND	M
Hs578t	U	M	M	M	U	U	U	ND
MCF7/Adr	ND	ND	M	ND	ND	U	ND	ND

U: unmethylated; M: methylated; U/M: partially methylated; D: biallelic deletion; ND: not done.

al. 1995, Davidson 2000). The presence of ER in breast tumors is a predictive marker for response to hormone therapy. However, up to one-third of breast carcinomas lack ER at the time of diagnosis and a proportion of cancers that are initially ER-positive lose ER during tumor progression (Hortobagyi 1998). Genetic alterations, such as homozygous deletion, LOH or ER gene mutation have not been reported to play a major role in loss of *ER* expression.

However, recent studies have shown that epigenetic alteration appears to play a role in inactivation of the gene (Ottaviano *et al.* 1994). The *ER* gene, located at chromosome 6q25.1, has a CpG island in its promoter and first exon regions. As demonstrated by Southern and methylation-specific PCR analyses, the *ER* CpG island is unmethylated in normal breast tissue and ER-positive tumor cell lines, such as MCF-7, T47-D and ZR75-1; it is methylated in ~50% of unselected primary breast cancers and most ER-negative cancer cell lines, e.g. MDA-MB-231, MDA-MB-435, MDA-MB-468, Hs578t and MCF-7/Adr (Table 2) (Lapidus *et al.* 1998). The *ER* CpG island methylation is associated with reduced or absent ER mRNA expression. The use of the methyltransferase inhibitors 5-aza-cytidine (5-aza-C) and 5-aza-dC, led to partial demethylation and restoration of ER mRNA expression, and synthesis of functional ER protein (Ferguson *et al.* 1995). Restored ER function was documented by eliciting estrogen response element-driven promoter activity from an exogenous plasmid as well as expression of the ER-responsive gene, PR.

In order to study *de novo* ER gene methylation *in vitro*, DNMT1 activity levels were measured in a panel of breast cancer cell lines. Expression of DNMT1 at both RNA and protein levels in ER-negative breast cancer cell lines is significantly elevated in ER-negative breast cancer cell lines compared with their ER-positive counterparts (Ottaviano *et al.* 1994). Detailed studies showed that DNMT1 expression was tightly correlated with S-phase fraction in ER-positive cells, while ER-negative cells expressed DNMT1 throughout the cell cycle, suggesting its expression is dysregulated in ER-negative breast cancer cells (Nass *et al.* 1999). The data also showed that DNMT1 and p21 expression are inversely correlated in the breast cancer cell lines examined. Studies using DNMT1 antisense constructs showed that decreased expression of DNMT1 protein is linked to increased p21 protein expression. Since p21 competes with DNMT1 for targeting to PCNA, the increased p21 may lead to inhibition of DNA replication and methylation in these tumor cells (Chuang *et al.* 1997).

A related question is whether an inactive chromatin structure mediated by HDAC is involved in *ER* gene silencing. In fact, inhibition of HDAC by HDAC inhibitor (A, trichostatin TSA) induced ER transcript by 5-fold in a panel of well-characterized ER-negative MDA-MB 231, Hs578t and MCF-7/Adr cell lines. This transcriptional reactivation was associated with increased sensitivity to DNaseI at the *ER* locus without alteration of the methylated CpG sites, suggesting that open chromatin structure is associated with ER expression even in the presence of *ER* CpG island methylation (Yang *et al.* 2000). Our findings identified a role for both DNA methylation and histone acetylation in the regulation of *ER* gene transcription.

PR *gene methylation*

The *PR* gene, located at chromosome 11q13, also has a CpG island in its first exon (Lapidus *et al.* 1996). The *PR* gene encodes two isoforms, hPR_A (79 kDa) and hPR_B (109 kDa), which differ in both their N-terminal sequences and biological activities. The hPR_B transcript is preferentially induced by ER while the hPR_A is not. Since ligand-bound ER is a major transcriptional activator of hPR_B gene expression, the presence of PR is indicative of functional ER. PR gene methylation has been demonstrated by Southern analysis in ~40% of PR-negative breast tumors and several PR-negative breast cancer cell lines (Table 2). A possible functional role for PR CpG island methylation is suggested by the observation that treatment of PR-negative MDA-MB-231 cells with 5-aza-dC in the presence of estrogen led to partial demethylation of the PR CpG island

and re-expression of PR gene. Co-treatment with both 5-aza-dC and a pure anti-estrogen, ICI 182,780, prevented PR reactivation, suggesting that demethylation alone is not sufficient to reactivate PR expression. Rather it appears that ER-mediated chromatin remodeling is essential and sufficient to activate PR gene expression even in the presence of a methylated PR CpG island (Ferguson *et al.* 1998).

RARβ2 gene methylation

RARα, -β and -γ and retinoid X receptors-α, -β and -γ are also members of the nuclear receptor superfamily (Minucci & Pelicci 1999). All six of these receptors are ligand-activated transcription factors (Chambon 1996). The RARβ gene, located at chromosome 3p24, appears to play an important role in limiting the growth of certain tumor types, including breast, lung and others. RARβ2 expression is often reduced or lost in breast cancer cells and they become resistant to induction by all-trans-retinoic acid (ATRA) (Swisshelm *et al.* 1994, Jing *et al.* 1996, Widschwendter *et al.* 1997). DNA methylation of the RARß promoter is believed to be one of the factors linked to RARβ2 downregulation in breast cancer (Widschwendter *et al.* 2000). RARβ promoter methylation has been demonstrated by Southern and methylation-specific PCR analyses in several RARβ2-negative human breast cancer cell lines and about one-third of unselected primary breast cancer specimens (Sirchia *et al.* 2000). It is not observed in normal breast tissue or HMECs. There is no apparent correlation with ER status. As with other methylated genes, treatment of RARβ2-negative cell lines with 5-aza-dC can partially re-induce RARβ2 transcripts. Of note, the HDAC inhibitor TSA can also reactivate RARβ2 expression in the presence of a methylated promoter, implicating inactive chromatin conformation as another possible regulatory process.

GSTP1 inactivation by methylation and its predisposition to genetic instability

Glutathione (GSH) and its corresponding cytosolic GSTs are involved in the detoxification pathway of xenobiotics and chemotherapeutic agents (Daniel 1993). They catalyze intracellular detoxification reactions by conjugating chemically reactive electrophiles to GSH, inactivating electrophilic carcinogens (Mannervik *et al.* 1985). The GSTs, encoded by several different genes at different loci, have been classified into α, μ, π and θ families. The π-class GST, encoded by the *GSTP1* gene, on chromosome 11, is of particular importance in breast cancer (Cairns *et al.* 1992, Gilbert *et al.* 1993). In cultured breast cancer cell lines an inverse relationship between *GSTP1* and *ER* gene expression has been reported, i.e. *GSTP1* was expressed in ER-negative but not in ER-positive lines (Table 2) although the underlying mechanism is unclear (Jhaveri & Morrow 1998). Treatment of the GSTP1-negative cell line MCF-7 with 5-aza-dC could induce mRNA expression and *de novo* synthesis of π-class protein. MSP-based studies of human tissues demonstrated that *GSTP1* promoter methylation is associated with gene inactivation in about 30% of primary breast carcinomas (Esteller *et al.* 1998). The detection of *GSTP1* methylation correlates with PR expression but there was no correlation with other clinical parameters such as the age at onset, histological type and grade, tumor size, nodal metastasis, DNA ploidy or ER status (Esteller *et al.* 1998). It is postulated that methylation-associated inactivation of *GSTP1* can result in adenine or guanine mutation by estrogen metabolites-DNA adduct formation and lead to genetic instability (Cavalieri *et al.* 1997).

BRCA1 methylation in sporadic breast cancer

The *BRCA1* gene, located at chromosome 17q21, is a well-known breast cancer susceptibility gene (Miki *et al.* 1994). Inhibition of *BRCA1* expression through antisense oligonucleotides increases the proliferation of normal and malignant mammary cells while overexpression of wild-type BRCA1 suppresses MCF-7 breast cancer cell tumorigenesis in mice. Inherited mutations in the *BRCA1* gene account for one-half of inherited breast carcinomas (Friedman *et al.* 1994). However, in contrast to other tumor suppressor genes, somatic mutations in this gene have not been reported, despite the high degree of LOH at the *BRCA1* locus in sporadic breast and ovarian cancer (Merajver *et al.* 1995). Since *BRCA1* transcript and protein are either absent or reduced in sporadic breast cancer, DNA methylation has been proposed as an alternative mechanism to inactivate *BRCA1* (Dobrovic & Simpfendorfer 1997, Magdinier *et al.* 2000). By Southern analysis of the *BRCA1* promoter region, methylation was detected in 11% of sporadic breast cancer cases and was inversely correlated with expression of both ER and PR (Catteau *et al.* 1999). A study with 194 primary breast carcinomas demonstrated that the *BRCA1* promoter is methylated in 13% of unselected primary breast tumors (Esteller *et al.* 2000*b*). *BRCA1* methylation was especially associated with medullary and mucinous subtypes. As expected, *BRCA1* was unmethylated in all normal tissues examined as well as in 21 breast cancer cell lines (Table 2). The methylation was present in two breast cancer xenografts with concomitant loss of gene transcript. In this study one allele is lost by LOH and the other is inactivated by aberrant methylation, thereby resulting in biallelic inactivation and loss of functional *BRCA1* gene product. Finally, *BRCA1* methylation is only observed in breast and ovary cancers but not in tumors of colon or liver or leukemia, supporting a tissue-specific event for the process. Using chromatin immunoprecipitation and endonuclease chromatin accessibility assays, transcriptional repression of *BRCA1* by cytosine methylation is also mechanistically linked to histone deactylation and inactive chromatin structure.

E-cadherin gene methylation and breast tumor progression

The *E-cadherin* gene, located at chromosome 16q22.1, encodes a cell-surface adhesion protein that is important in maintaining homophilic cell–cell adhesion in epithelial tissues (Ilyas & Tomlinson 1997). Considerable evidence shows that loss of expression and function of E-cadherin protein contributes to increased proliferation, invasion and metastasis in breast cancer (Oka *et al.* 1992). Classical mutations and deletions clearly play a role in loss of the E-cadherin expression and function (Cleton-Jansen *et al.* 1994, Risinger *et al.* 1994). However, several studies demonstrate that epigenetic silencing of the *E-cadherin* gene by 5′CpG methylation occurs in some human breast cancer cell lines (Table 2) as well as about 50% of unselected primary breast cancers (Graff *et al.* 1995, Hiraguri *et al.* 1998). Its loss of expression is associated with tumor metastatic progression and decreased patient survival (Bringuier *et al.* 1993). Our recent work demonstrated that hypermethylation of the *E-cadherin* CpG island was evident in about 30% of ductal carcinomas *in situ* and increased significantly to nearly 60% of metastatic lesions (Nass *et al.* 2000), suggesting a role for this process in tumor progression.

Methylation and inactivation of *TIMP-3* gene

TIMP-3 belongs to a family of molecules that inhibit the proteolytic activity of the MMPs (Gomez *et al.* 1997, 1999). This protein can suppress primary tumor growth via its effects on tumor development, angiogenesis, invasion and metastasis (Uria *et al.* 1994). Methylation of its 5′CpG island has been associated with the loss of *TIMP-3* expression at both transcript and protein levels in several tumor types (Bachman *et al.* 1999). The *TIMP-3* promoter region is methylated in ~30% of human breast cancer cell lines as well as ~30% of primary breast tumors (Bachman *et al.* 1999). *TIMP-3* gene methylation has been associated with its loss of expression as its expression could be restored by 5-aza-dC treatment, again supporting a role for epigenetic mechanism in *TIMP-3* gene regulation.

Clinical implications of epigenetic regulation in breast cancer

Recognition of the important roles that DNA methylation and histone deacetylation play in gene expression in malignancy including breast cancer has led to consideration of how these findings can be exploited clinically. Two areas for exploration are the use of methylated markers for detection and prognosis, and the application of DNMT and HDAC inhibitors therapeutically to re-express silenced tumor suppressor and growth inhibitory genes.

Methylated markers for detection and prognosis

One of the tenets of epigenetic regulation is that CpG islands are generally unmethylated in normal adult tissues with rare exceptions. In contrast, as noted earlier, hypermethylation of various gene promoters is a common feature of malignant cells and these changes can occur early in the progression process. For example, about one-third of ductal carcinoma *in situ* lesions demonstrated methylation of the *E-cadherin* or *ER* promoter CpG islands (Nass *et al.* 2000). The availability of PCR-based strategies to assess methylation changes in minute quantities of biological materials raises the possibility that identification of methylated alleles might serve as a means of molecular risk assessment or detection (Herman *et al.* 1996). Indeed the ability to detect methylated genes in serum, urine, bronchoalveolar lavage fluid and lymph nodes derived from individuals with various types of malignancies has been described (Esteller *et al.* 1999*b*). In some cases methylated changes were also detected in samples banked well before the diagnosis of malignancy, raising the possibility that this type of analysis could facilitate risk assessment or early diagnosis strategies. One potential application in breast cancer would be the assessment of methylated markers using nipple duct lavage fluids or fine needle aspirates of the breast, as it could be hypothesized that their presence would predict a higher likelihood of subsequent breast cancer development. Other applications could include molecular staging of sentinel lymph nodes or bone marrow aspirates. Such studies will first require the development of a panel of methylated markers that would together identify virtually all breast cancers but that are unmethylated in normal tissues (Evron *et al.* 2001).

Finally, another possible clinical application for gene-specific methylation is prediction of prognosis or treatment outcome in certain cancers (Weinstein 2000). For example, the DNA-repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) inhibits the ability of alkylating agents to kill tumor cells; loss of its expression by methylation might be expected to sensitize tumor cells to this class of agents. Indeed, methylation of MGMT is correlated with increased overall and disease-free survival and improved response to the alkylating agent carmustine in glioblastoma patients (Esteller *et al.* 2000*a*). Therefore, MGMT promoter methylation is a marker of good prognosis and predicts response to chemotherapy for glioblastoma patients.

DNMT and HDAC inhibitors for therapy of breast cancer

Although heritable, epigenetic changes are potentially reversible. Therefore, the prospect of intervening to reverse these changes as a possible means of reverting the malignant

phenotype is an attractive one. The availability of DNMT and HDAC inhibitors makes this a testable strategy.

DNMT inhibitors

The classic DNMT inhibitors, 5-aza-C and 5-aza-dC are cytosine analogs that are incorporated into replicating DNA (Jones & Taylor 1980, Jones 1985). The subsequent formation of covalent adducts between DNMT and 5-aza-C-substituted DNA irreversibly inactivates DNMT (Santi *et al.* 1984, Ferguson *et al.* 1997). One potential consequence then is the reactivation of previously methylated genes that have been transcriptionally silent. These analogs have been used clinically for treatment of patients with hemoglobinapathies, myelodysplasia and leukemia (Rivard *et al.* 1981, Ley *et al.* 1983). The clinical benefit observed has been associated with reactivation of previously silenced genes as, for example, the observation that 5-aza-treated sickle cell patients have improved hemoglobin and increased fetal hemoglobin (Nienhuis *et al.* 1985). Such strategies could potentially be applied to epithelial malignancies as well. For example, a gene microarray analysis of the effect of 5-aza-dC on HT29 colon cancer cells identified members of the interferon (IFN) response pathway as re-expressed genes (Karpf *et al.* 1999). Follow-up studies showed that 5-aza-dC treatment of these cells *in vitro* sensitized them to growth inhibition by exogenous IFN-α2a. Thus unmasking of new therapeutic targets may be enhanced.

The elevated level of DNMT activity in cancer has also prompted development of more specific agents like antisense oligonucleotides to DNMT1. *In vitro* studies have shown that antisense treatment of human T24 bladder cancer cells and A549 non-small lung carcinoma cells can cause reduced DNMT1 levels, demethylation of the *p16*^{INK4A} gene promoter, re-expression of p16^{INK4A} protein, accumulation of the hypophosphorylated form of the Rb protein, increase in p21$^{WAF/CIP1}$, and cell growth arrest (Fournel *et al.* 1999). Thus, this specific targeting of DNMT1 can activate silent tumor suppressors.

HDAC inhibitors

Several structural classes of HDAC inhibitors have been identified. Phenylbutyrate, a short chain fatty acid that inhibits HDAC at millimolar concentrations and is approved for clinical use by the US Food and Drugs Administration (FDA), is an example of the first class (Carducci *et al.* 1996). The second class includes the hydroxamic acids, trichostatin A (Yoshida *et al.* 1990) and suberoylanalide hydroxamic acid (SAHA) (Richon *et al.* 1998). Nanomolar concentrations of TSA have been used widely in *in vitro* systems to inhibit HDAC activity and modify gene transcription. SAHA is also active in *in vitro* models at micromolar concentrations. It induces terminal cell differentiation with milk protein synthesis in MCF-7 cells (Richon *et al.* 1998). Further, its administration reduced the incidence of N-methyl-nitrosourea-induced mammary carcinoma in rats without apparent toxicity (Desai *et al.* 1999). Phase I trials of SAHA are in progress (Marks *et al.* 2000). The third class of HDAC inhibitor, benzamide derivatives like MS-27–275, also showed marked activity against human tumor xenografts in mice (Saito *et al.* 1999). The fourth class includes cyclic tetrapeptides containing a 2-amino-8-oxo-9,10-epoxy-decanoyl (AOE) moiety such as trapoxin A (Kijima *et al.* 1993), while FR 901228 and apicidin are representative of the fifth class of cyclic peptides that lack an AOE moiety (Nakajima *et al.* 1998).

Combination strategies

Combined therapies may ultimately offer the best antineoplastic approach. If successful, such strategies could utilize agents that alone are ineffective but together result in the desired biological outcome. For example, sequential treatment of colon cancer cells with a demethylating agent followed by an HDAC inhibitor led to reactivation of multiple target genes whose expression was not affected by either agent alone (Cameron *et al.* 1999). Our own studies show that combined therapy of MDA-MB-231 cells with 5-aza followed by TSA results in re-expression of ER at lower doses than those needed with either single agent (Yang *et al.* 2001). Thus, it may be possible to use lower doses or shorter exposure durations to achieve the appropriate molecular effects, thereby increasing therapeutic effects, minimizing toxicity and improving ease of administration.

Alternatively, use of an 'epigenetic modifier' may enhance the response to another type of agent. The use of 5-aza and IFN in colon cancer cells was mentioned above. A human proof of principle for this general strategy is seen in the report of a patient with acute promyelocytic leukemia resistant to ATRA who developed a sustained remission after concomitant treatment with phenylbutyrate and ATRA (Warrell *et al.* 1998).

Conclusions

Taken together, substantial evidence demonstrates the importance of epigenetic mechanisms in the transcriptional regulation of critical tumor suppressor and growth regulatory genes in breast cancer. These genes include those that play crucial roles in DNA repair, cell cycle regulation, cell growth and cell–cell adhesion. These changes, along with the intrinsic ability of 5meC to function as a mutagen and the negative effects of dysregulated DNMT1 activity, can all contribute to breast cancer tumorigenesis and/or progression. A better understanding of epigenetic regulation of gene expression in a gene-specific and tissue-specific fashion will help efforts to modulate gene expression selectively with the ultimate goal of improved breast cancer prevention and therapy.

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Specific Inhibition of DNMT1 by Antisense Oligonucleotides Induces Transcriptional re-expression of Estrogen Receptor α in ERα-negative breast cancer cell lines

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Abstract

Estrogen and progesterone and their receptors (ERα and PR) play major roles in the development and function of the mammary gland as well as in breast cancer biology and treatment. However, 25-30% of human breast cancers lack ERα and PR expression and respond poorly to hormonal treatment. Absence of ERα and PR expression is associated with lack of transcript, methylation of the CpG islands in the promoter regions of these genes, and increased DNMT activity and DNMT1 protein. Treatment of ER-negative human breast cancer cell lines with a non-specific DNMT inhibitor 5-azacytidine led to reactivation of functional ER protein. This study addresses the hypothesis that specific inhibition of DNMT1 by antisense oligonucleotides (DNMT1 ASO) is sufficient to re-express ERα and PR genes in ER-/PR- human breast cancer cell lines. MDA-MB-231 and Hs578t cells were transfected with 100 nM and 150 nM DNMT1ASO for three consecutive days respectively. Significant growth reduction was observed after 48 hr, and this inhibition was more obvious after 72 hr or 96 hr. DNMT1 expression was blocked after exposure to DNMT1 ASO as detected by Western blot whereas mutated DNMT1 ASO had no effect. Transcriptional re-expression of ERα and PR as well as other methylation-silenced genes such as cyclinD2 (CD2) and retinoic acid receptor β (RARβ) were observed in MDA-MB-231 cells after this multiple DNMT1 ASO treatment. ERα protein expression was observed in MDA-MB-231 cells 96 hours after ASO treatment. However, methylation specific PCR indicated that ERα promoter CpG islands were only partially demethylated. Other mechanisms such as disruption of repressive transcription complex involving DNMT1, demethylation of other portions of ER promoter, etc. need to be explored in the future studies. Our results suggest that specific DNMT1 inhibition alone is sufficient to re-express ERα in human breast cancer cell lines, implying that DNMT1 ASO might be useful in supportive treatment of ER-negative human breast cancer with anti-estrogen regimen. Supported by DAMD 17-98-1-8116 and NIHR01 CA78352.

Introduction

Estrogen plays a major role in the initiation, progression and treatment outcome of breast cancer. Tumors expression ERα are sensitive to hormone therapy, such as tamoxifen (McGuire WL,1979). However, up to one-third of primary breast cancers are hormone-independent because they lack ERα (Hortobagyi G, 1998). Moreover, some ERα-positive patients will convert to an ERα-negative state and lost their sensitivity to endocrine therapy during the course of disease (Biswas DK, 1998). So far, no significant genetic alterations such as insertions, deletions, rearrangements, or point mutations within the ER gene have been reported that would explain the loss of ER function in ERα-negative breast cancers (Reviewed in Lapidus, 1998). Recent findings suggest that epigenetic changes like abnormal methylation of CpG islands have been associated with inhibition of expression of a variety of critical genes, including ERα, RARβ, cyclinD2, p16, Rb, E-cadherin (Ferguson 1995, Sirchia 2000, Sara 2000, Herman, 1995; Ohtani-Fujita, 1993; Yoshiura, 1995). The CpG island within promoter and first exon of the ERα gene is extensively methylated in ERα-negative breast cancer cells but remains unmethylated in normal breast tissue and many ERα-positive tumors and cancer cell lines (Ottaviano, 1994; Lapidus, 1996). The functional importance of this finding is demonstrated by the fact that treatment of ERα-negative breast cancer cells with non-specific demethylating agents like 5-aza-2'-deoxycytidine (aza) leads to re-expression of ERα mRNA and functional protein (Ferguson, 1995). Although PR also has a typical CpGisland in its promoter and first exon, induction of PR gene expression by ligand-bound ER does not require demethylation of the PR CpG island (Ferguson, 1998). Another epigenetic regulation via generation of inactive chromatin through deacetylation of lysine groups of histones by histone deacetylases (HDACs) has been demonstrated that can regulate expression of genes like ERα. Using the HDAC inhibitor, trichostatin A (TSA), transcriptional activation of ERα in ERα-negative human breast cancer cells was observed without change in methylation status, suggesting that histone acetylation is an alternative pathway in epigenetic regulation of ERα gene expression (Yang, 2000, Yan 2001). Recent studies demonstrated a direct link between the two processes of DNA methylation and histone acetylation by showing that DNMT1 and HDAC can form a repressive transcription complex with other associated protein sat replication foci (Rountree MR, 2000; Robertson KD, 2000).

Antisense macromolecular drugs are designed to base pair between relatively short oligonucleotides (twelve to eighteen residues) and complementary sequences in mRNA to provide highly specific inhibition of genes of interests. Antisense oligonucleotides can inhibit or modify gene expression via several different mechanisms 1) the RNA (mRNA)/DNA (antisense oligonucleotides, ASO) duplex formed may trigger the activation of Rnase H, the cellular enzyme that degrades the RNA component; 2) block translation of the mRNA into protein if the ASO targets at the translation initiation site or at the 5' cap region; 3) disrupt RNA secondary structures that are necessary for RNA stability. (Crooke,1999; Bennett, 1998; Gewirtz, 1998). Antisense oligonucleotides can also be used to alter, rather than inhibit, gene expression through interrupting mRNA transcript splicing patterns where ASOs directed to the mutant splice sites can force the splicing machinery to use the wild-type splice donor and acceptor sites, thus resulting in correctly spliced mRNA and functional protein (Sierakowska, 1996, Lacerra 2000). Since approximately 15 percent of genetic diseases are caused by defects in splicing, ASO might be a unique alternative way to correct these genetic defects. DNMT1 ASO98 used in this study is a second generation ASO that contains phosphorothiate backbone modifications but also contain 2'-O-methyl modifications to the sugar moiety of the four nucleotides on both the 5' and 3' end of the molecule. This 2'-O-methyl second generation modification serves to increase half-life in vivo further, increase affinity of the antisense molecule for its mRNA target and to reduce side effects in vivo (Inoue, 1987, Agrawal). ASO98 targeted to the 3-UTR (untranslated region) of the DNMT1 mRNA. Because antisense activity depends on hybidization to the mRNA target, control ASO207 molecules containing mismatched bases that do not bind with high affinity to the target was designed and tested to control for potential non-specific effects of this class of compounds in a given biological system. Our purpose is to investigate the role of DNMT1 in epigenetic regulation of ER expression by use of specific DNMT1 inhibitor --- ASO98.

Materials and Methods

Cell lines, Reagents and ASO Treatment.

ASO98 (5' –mUmUmCmATGTCAGCCAAGGmCmCmAmC –3') and its mutant control ASO207 (5' – mUmU*AmATGT*AA*CC*TAAG*TmCmCmAm*A- 3') were designed based on an IND application and ordered from the Midland Certified Reagent Company (Midland, TX). Two ER-negative cell lines, MDA-MB-231 and Hs578t, were treated with ASO98 or mutant control ASO207 by use of a repetitive

treatment strategy. Cells were seeded at -24 hr at a density of 6 x 10^5 cells per 100 mm tissue culture dish, lipofected according to manufactures' instruction (Gibco, Carlsbad, CA) at hours 0, 24, and 48 hr with ASO98, ASO207 or vehicle only (Sham) and then harvested at 96 hr for isolation of DNA, RNA and protein.

RNA Isolation and RT-PCR Analysis of ER Expression.

Total cellular RNA was isolated from cell lines with TRIzol reagent according to the recommendations of the supplier (GibcoBRL, Rockville, MD). RNA (3ug) was reversibly transcribed by M-MLV reverse transcriptase (GibcoBRL) using $OligoDT_{15}$ primer (Promega, Madison, WI). An aliquot of synthesized cDNA was used for PCR amplification of ER and the constitutively expressed house-keeping gene β-actin. Specific sense and anti-sense PCR primers used for amplifications across the seventh intron of ER and the first intron of β-actin genes, yielding 470 bp and 400 bp of PCR products respectively, as described previously (Ferguson, 1995). After 35 cycles of amplification the PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The RT procedures for PR, CD2, DNMT1, DNMT3A, DNMT3B, RARβ are very similar to that for ER except using different PCR programs (Furguson ; Evron 2001; Robertson Nucl.Acids. Res 2000; Widschwendter et al. 2000).

Genomic DNA Isolation and MSP Analysis of ER CpG Island. DNA was isolated from cell lines by solubilization in a SDS/proteinase K solution, followed by phenol/chloroform extractions and ethanol precipitation. DNA was quantified by determination of OD_{260}. Isolated DNA was subjected to modification by sodium bisulfite to convert unmethylated cytosines but not methylated cytosines to uracil as previously described (Herman 1996). Methylation status of the bisulfite-modified DNA at the ER locus was characterized by methylation specific PCR using previously described method (Lapidus, 1995).

Western Blot Analyses and DNMT Activity. Western blot analyses were used to demonstrate the reduction of DNMT1 protein expression and ER expression with ASO treatment. DNMT1 antibody was a gift from Dr. William Nelson, ER antibody was ordered from Santa Cruez, and actin antibody was from Sigma. DNMT activity was measured as described previously (Vertino, 1996).

Growth inhibition induced by ASO. Cells were harvested at 24, 48, 72 and 96 hours after ASO treatment and cell numbers were assessed by use of Coulter Particle Counter ( ).

Results and Discussion

A major challenge in improving the treatment of breast cancer is understanding and overcoming resistance to endocrine therapy. About one quarter to one third of human breast cancers lack estrogen receptor α (ER) and progesterone receptor (PR), a phenotype associated with poor response to endocrine therapy. However, the molecular mechanisms underlying this lack of ER and PR expression are poorly understood. Recent findings suggest that epigenetic changes may be important for tumor initiation or progression. Abnormal methylation of CpG islands has been associated with inhibition of expression of a variety of genes, including ER in breast cancer. A CpG island in the promoter region of the ER gene is extensively methylated in ER-negative breast cancer cells, but is unmethylated in normal breast cells. Furthermore, expression of a major enzyme that catalyzes cytosine methylation, DNA methyltransferase 1 (DNMT1), is significantly elevated in ER negative breast cancer cell lines compared to ER-positive lines. Treatment of ER-negative cell lines with the DNMT inhibitor, 5-azacytidine, leads to reexpression of functional ER protein. Over the past three years, the discovery of the link between DNMT1 and HDAC in epigenetic silencing stirred up much excitement (Rountree 2000; Robertson 2000). Those sutdies suggest that besides methylating DNA, DNMT1 plays an important epigeneitc regulatory role by forming a crucial transcription repressive complex at DNA replication foci. In addition, another group of DNMTs, DNMT3A and DNMT3B, was isolated by database search. Both DNMT3A and 3B could methylate hemimethylated and unmethylated DNA with equal efficiencies, making them potential candidates for the de novo methyltransferase (Okano M 1998, Nature Genet. 19, 219-220). The goal of this study was to assess the precise role of DNMT1 in regulating ER expression in ER negative human breast cancer cell lines using a specific DNMT1 antisense oligonucleotide (ASO) treatment.

First, we set out to determine the growth inhibition of DNMT1 ASO on human breast cancer cell lines. Two ER-negative cell lines, MDA-MB-231 and Hs578t, were seeded at -24 hr at a density of 6×10^5 cells per 100 mm tissue culture dish, lipofected at hours 0, 24, and 48 hr with ASO98. Cell growth with mutant control ASO207, vehicle only (Sham) or growth without any treatment (Untreated) were used as controls. The ASO concentration used were 100 nM for MDA-MB-231 cells and 150 nM for Hs578t cells based on their dose response studies (Data not shown). Adherent cells were harvested and counted by use of Coulter counter at 0, 24, 48, 72 and 96 hr. As shown in Fig. 1, significant growth reduction was

observed for both ASO98-treated cell lines after 48 hr, and this inhibition was more obvious after 72 hr or 96 hr. Some growth inhibition was also observed for both mutant ASO207-treated cell lines, suggesting non-specific effects of ASO. It has been well documented in antisense technology that phosphorothioate oligonucleotides can bind to proteins and thus cause many biological effects unrelated to their antisense action. Growth inhibition is one of its consequences (Gewirtz, 1999). DNMT1 expression was blocked within 48hr-96 hr of exposure to DNMT1 ASO98 as detected by Western blot whereas mutant DNMT1 ASO207 had no effect (Fig. 2). RT-PCR analysis showed DNMT1 mRNA was blocked after ASO98 treatment but not the mutant ASO207 (Fig. 3). The specificity of DNMT1 ASO98 was also shown by its no effects on DNMT3A and 3B expression (Fig. 3). Reexpression of ER was observed in both cell lines after the ASO98 treatment as detected by RT-PCR (Fig. 3). Reexpression of other breast cancer related genes, such as PR, Retinoic Acid Receptor β (RARβ), and cyclin D2 (CD2) mRNA were detected in MDA-MB-231 cells (Fig. 4). Fig. 5A showed the DNMT activity was significantly decreased (approximately 95%) in MDA-MB-231 cells in a pool of four individual experiments after ASO98 treatment. Reexpression of ER protein in the same cell line with ASO98 treatment was observed in Western Blot analysis as shown in Fig 5B. Although mutant ASO207 treatment also caused 25% decrease in DNMT activity, no evidence of ER protein reexpression was observed (Fig. 5B). **(Fig 5C functional study)** Finally, ERα promoter CpG methylation status was assessed by methylation specific PCR (MSP) – a technique designed to analyze methylation of CpG dinucleotides across the entire CpG island (Fig. 6A). Four sets of primers (ER1, 3, 4 and 5) were used to amplify overlapping fragments of CpG island in ERα promoter region. This series of ER promoter "MSP characterization" method has been shown reliable to predict the ER methylation status in our previous study ( ). ERα-positive MCF-7 cells were used as unmethylated control. Distilled water instead of bisulfite-treated genomic DNA was used as MSP-negative control. Cells treated with ASO98 showed the possibility of partial demethylation pattern in ER5 primer set (Fig. 6B). However we did not see any demethylation evidence in other three sets of MSP (data not shown). One explanation for those results might be that the re-expression of ERα by specific DNMT1 inhibition do not rely exclusively on CpG demethylation. Recent studies demonstrated that disrupting the *DNMT1* gene through homologous recombination in human colorectal carcinoma cells could markedly decrease cellular DNMT activity, however there was only a 20% decrease in overall genomic

methylation (Rhee, 2000). These studies suggest that DNMT1 could neither be the sole enzyme contributing to the methylation status of entire human genome nor the abnormal methylation found in cancer-related genes. Whether and how much of other methylating enzymes involved in this DNA epigenetic modification is currently under extensive investigation. Functional studies indicate that DNMT1 can form a repressive complex at replication foci with histone deacetalases (HDACs) and a newly identified protein, DMAP1 (DNMT1 associated protein), at the non-catalytic amino terminus of DNMT1. This complex can inhibit transcription initiation of relevant genes. Therefore one of mechanistic hypotheses for our DNMT1 ASO study is that the re-expression of ERα is through disruption of the repressive transcription complex at ER promoter region. Overall, our DNMT1 ASO study suggests that ASO can inhibit DNMT1 and cause reexpression of ER as well as other epigenetic regulated genes in human breast cancer cell lines. These preliminary observations shed light on the possibility of using DNMT1 ASO as an adjuvant agent in anti hormone medication regimen for patients with ER negative human breast cancer or other cancers in which epigenetic regulation are confirmed to be a crucial factor.

Figure legends

Fig.1: Growth inhibition induced by DNMT1 antisense oligo (ASO) treatment in MDA-MB-231 and Hs578t human breast cancer cells. Cells were grown in the presence of no treatment (Untreated); vehicle only (Sham); DNMT1missense oligo treatment (ASO207 100 nM x 3 days for MDA-MB-231 cells, 150 nM x 3 days for Hs578t cells); and DNMT1 ASO98 treatment (100 nM x 3days for MDA-MB-231 cells, 150 nM x 3 days for Hs578t cells). Arrows represent days of lipofectin treatment. Adherent cells were counted by Coulter Counter. Each point shows mean ±SEM for triplicate determinations in a representative experiment.

Fig.2: Specific inhibition of DNMT1 protein expression in two ERα-negative cell lines treated with ASO98. A polyclonal antisera raised against human DNMT1 was used in this Western analysis. The

expected 200 kDa band of DNMT1 was detected in Untreated, Sham and ASO 207 (missense oligo control) treated cells, but was markedly reduced after ASO98 treatment (100 nM x 3 days for MDA-MB-231 cells or, 150 nM x 3 days for Hs578t cells), but ASO207 treatment was not affected. Actin was used to show the equal loading of protein lysates.

Fig.3: RT-PCR analysis of DNMT1 and ERα mRNA expression in two ER negative cell lines, MDA-MB-231 and Hs578t, after DNMT1 Antisense Oligo (ASO98) treatment. DNMT1 mRNA transcript was blocked after ASO98 treatment (100 nM x 3 days for MDA-MB-231 cells, 150 nM x 3 days for Hs578t cells), and ERα mRNA re-expression was noted in both cell lines. No effect was seen on DNMT1 and ER α expression in untreated, sham, or ASO207 treated. DNMT3A and 3B were studied to show the specificity of DNMT1 ASO treatment. β-actin RT-PCR product provides a control for the amount of intact RNA used in the reaction.

Fig.4: RT-PCR analysis of RARβ, Cyclin D2 and PR expression in ER negative MDA-MB-231 cells after DNMT1 antisense oligo (ASO98) treatment RAR β, CyclinD2 and PR mRNA re-expression was noted after ASO98 treatment, but not in untreated, sham, or ASO207-treated cells. β-actin RT-PCR product provides a control for the amount of intact RNA used in the reaction.

Fig.5A: DNMT activity inhibition by ASO98 in MDA-MB-231 cells. Each bar represents a pool of four individual cell culture dishes. DNMT activity for each treatment was documented as the percentage of control DNMT activity of Hct1116 cells. Individual activity was normalized by the amount of protein in each sample.

Fig.5B: Western Blot Analysis of ERα reexpression in MDA-MB-231 after ASO treatment. MCF-7 cells were used as a positive control. Actin was used to show the equal loading of proteins in each sample.

Fig.6A: Map of ERα CpG island and locations of PCR products generated by use of specific ERα MSP primers. Box represents exon 1 of ERα gene.

Fig.6B: MSP analysis for the ERα CpG island in DNMT1 ASO-treated ERα-negative MDA-MB-231 cells. Untreated, Sham, and ASO207 (missense oligo) were used as methylated controls. ERα-positive MCF-7

cells were used as unmethylated control. Distilled water instead of bisulfite-treated genomic DNA was used as MSP-negative control. ASO98 treatment may be associated with partial demethylation of the ER CpG island. u: unmethylated products; m: methylated products.

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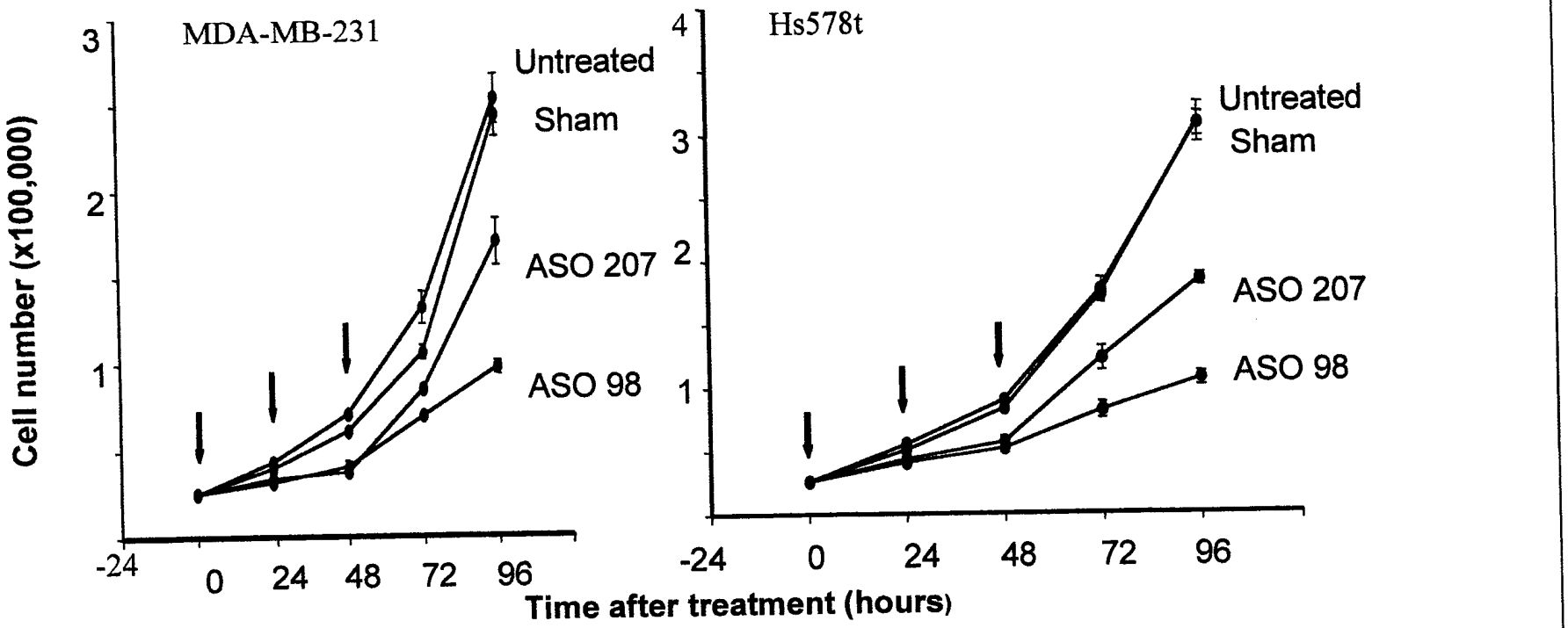

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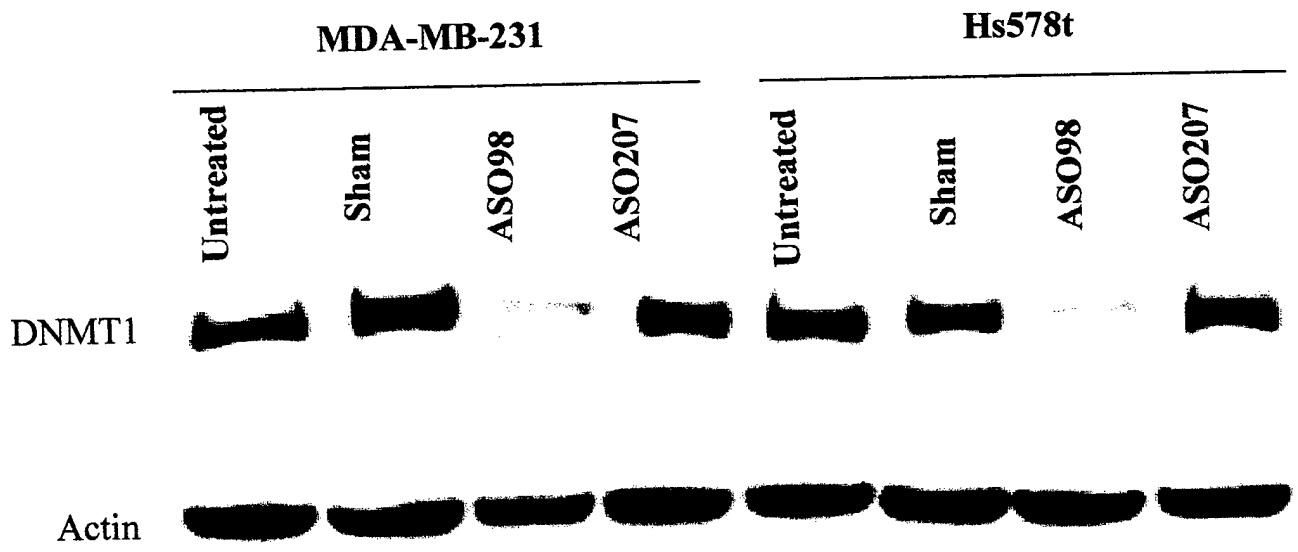

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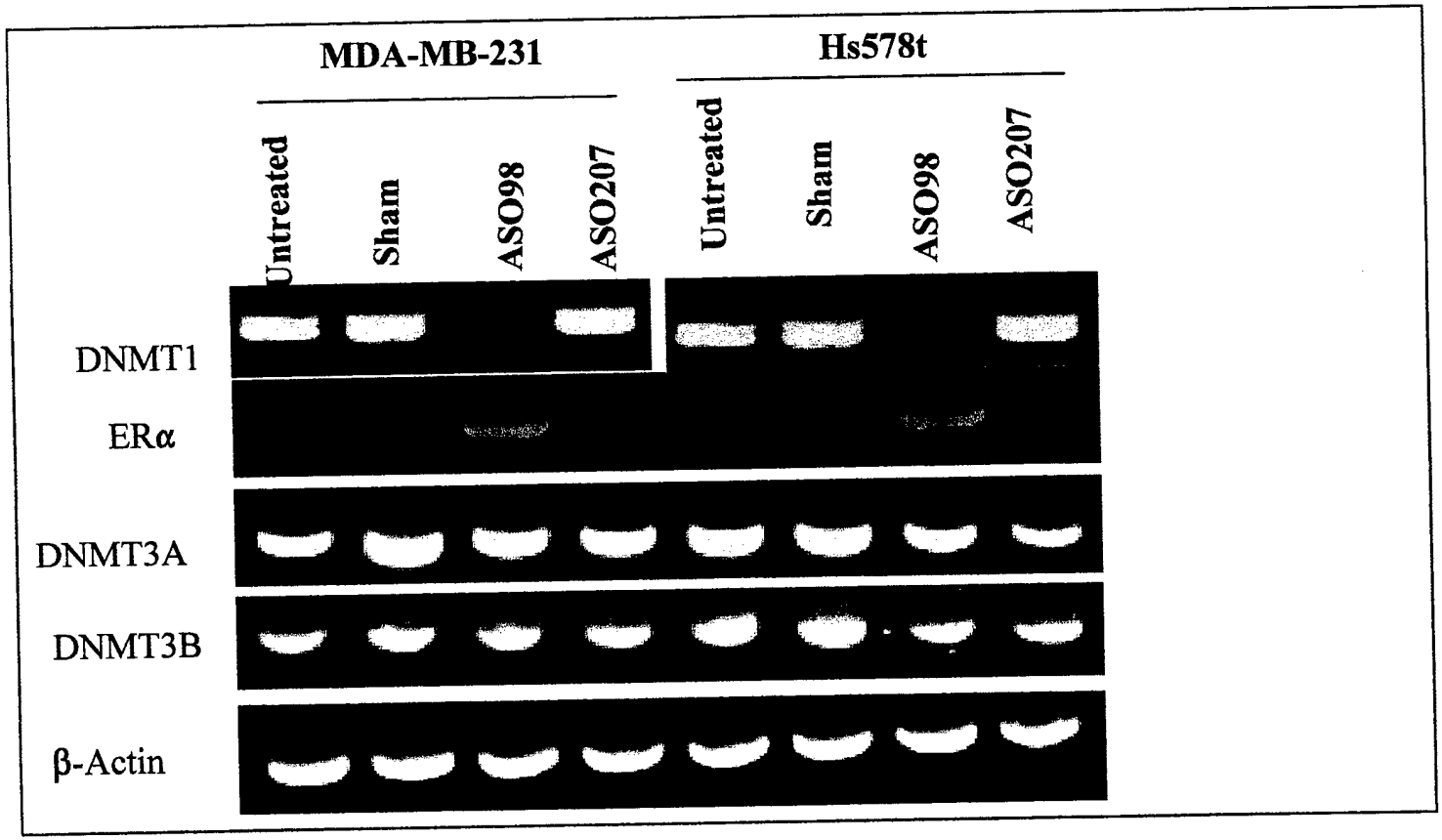

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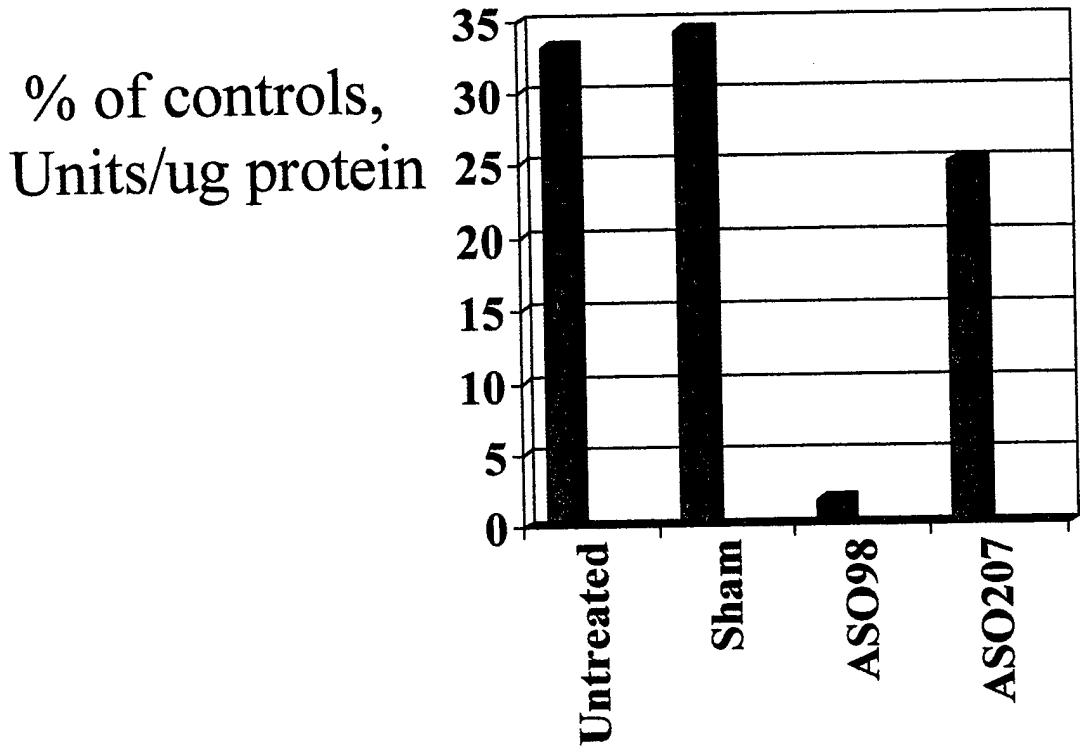
DNMT Activity after ASO Treatment in MDA-MB-231 Cells



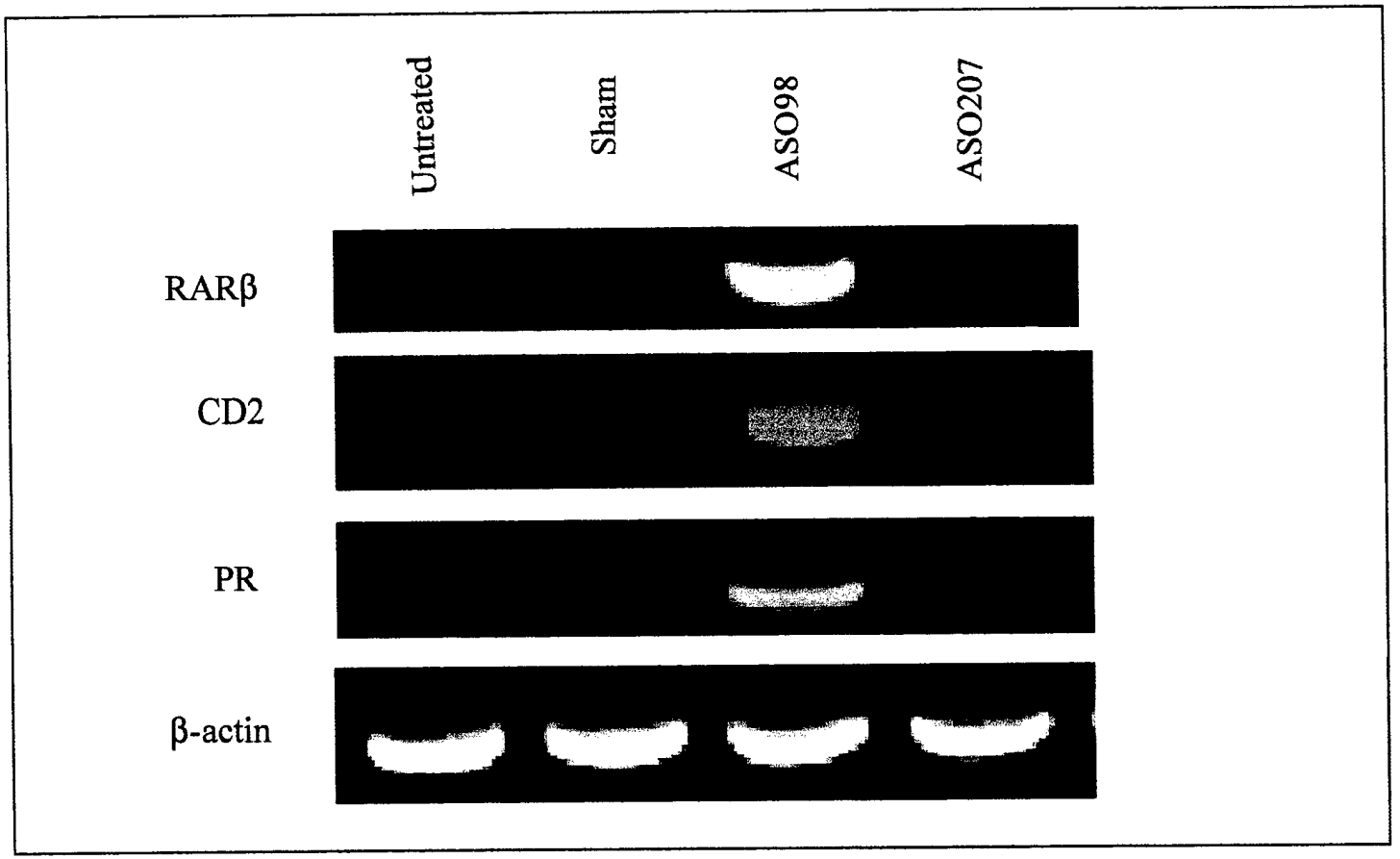


Fig.5: RT-PCR analysis of RARβ, Cyclin D2 and PR expression in ER negative MDA-MB-231 cells after DNMT1 antisense oligo (ASO98) treatment β-actin RT-PCR product provides a control for the amount of intact RNA used in the reaction. RAR β, CyclinD2 and PR mRNA re-expression was noted afterASO98 treatment.

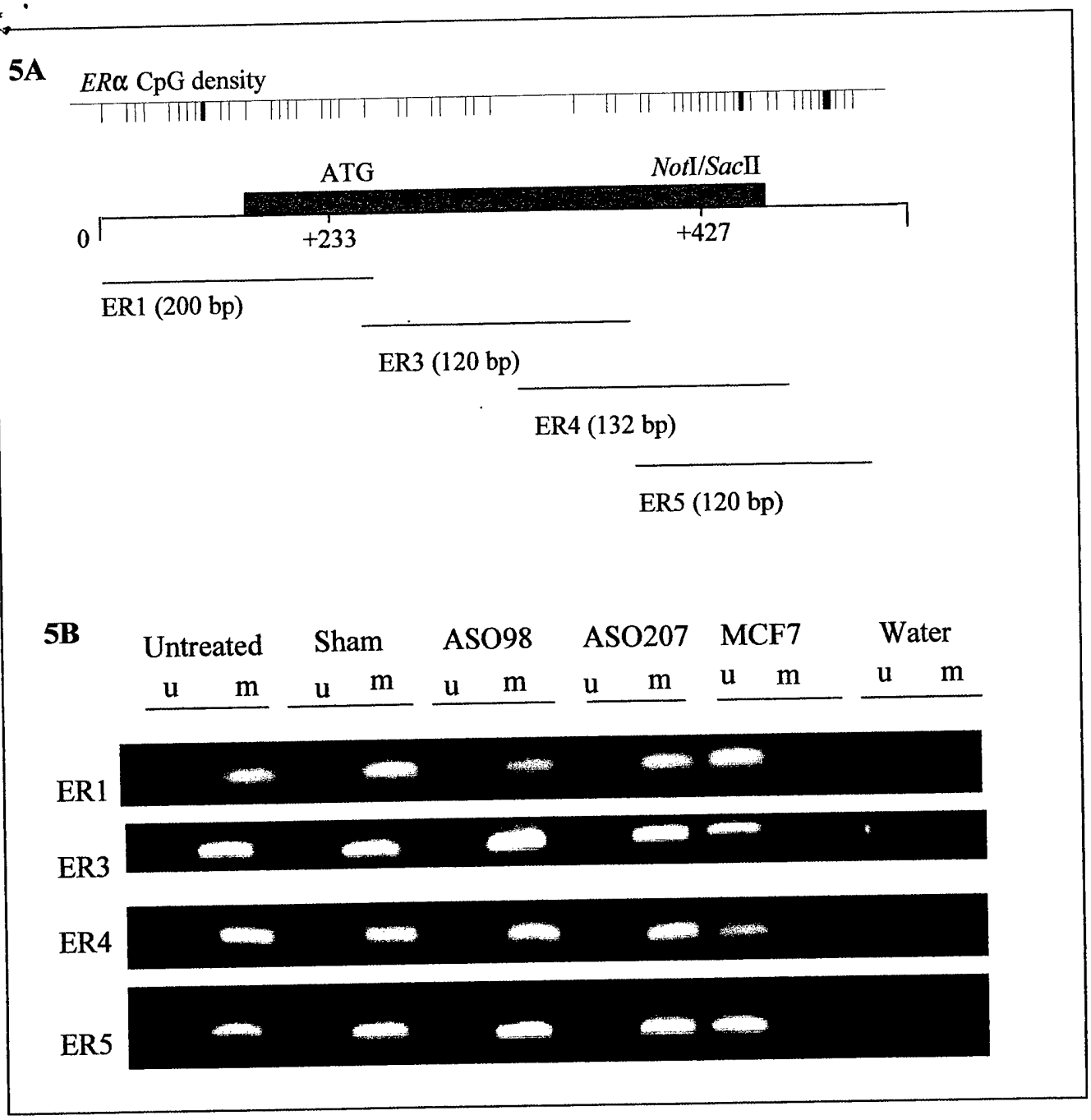


Fig.5A: Map of ERα CpG island and locations of PCR products generated by use of specific ERα MSP primers. Box represents exon 1 of ERα gene.
Fig.5B: MSP analysis for the ERα CpG island in DNMT1 ASO-treated ERα-negative MDA-MB-231 cells. Untreated, Sham, and ASO207 (missense oligo) were used as methylated controls. ERα-positive MCF-7 cells were used as unmethylated control. Distilled water instead of bisulfite-treated genomic DNA was used as MSP-negative control. ASO98 treatment may be associated with partial demethylation of the ER CpG island. u: unmethylated products; m: methylated products.

Western Blot Analysis